STUDIES ON THE OVINE MAST CELL : HETEROGENEITY AND INVOLVEMENT IN CUTANEOUS INFLAMMATION

GORDON HUNTER STURE

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH
1996

DECLARATION

I hereby declare that:-

(i) This thesis has been composed by myself.

(ii) It has not been accepted in any previous application.

(iii) The work described has been carried out by myself or, where jointly, this fact has been acknowledged.

ACKNOWLEDGEMENTS

I am indebted to Dr. John Huntley (Moredun Research Institute [MRI], Edinburgh) for his help, humour, encouragement and support during the course of these studies, for which I am particularly grateful. I am also equally indebted to my supervisor Professor Hugh Miller (Department of Veterinary Clinical Studies, Royal Dick School of Veterinary Studies [RDSVS], The University of Edinburgh) for advice, support and encouragement. At the MRI, I am grateful to Miss. Anne MacKellar for help and advice with respect to both tissue culture techniques and to histological processing and staining. Thanks are also due to Mr. George Newlands for help with FPLC techniques and general technical advice and to Mrs. Lesley Inglis for instruction on antibody purification by affinity chromatography. I am especially grateful to Dr. Gary Entrican for the generous gift of recombinant ovine interleukin-3, to Dr. Gordon Moon for performing endotoxin assays and to Mr. David Hitchcock of Biometrics and Statistics Scotland for advice on experimental design and data analysis. I must also thank the staff of the Clinical Department, MRI for their attention to animal care and husbandry during these studies.

At the RDSVS I am indebted to Dr. Alan Pemberton for technical advice and for preparation of SMCP and heat-inactivated SMCP. Thanks are also due to Mrs. Elizabeth Thornton for technical advice and for monitoring my initial attempts at tissue culture, and to Dr. Cheryl Scudamore for advice on statistics and general laboratory practice.

I am also grateful to Mr. Bob Munro (RDSVS) and to Mr. Brian Easter (MRI) for their technical expertise and help with photography and photomicrography, and to Mr. S.Mitchell of the Department of Preclinical Veterinary Studies, RDSVS for help with electron microscopy.

A general vote of thanks must also go to members of the Immunopathology Department (MRI) and to the members of Prof. Millers mast cell group (RDSVS) for making these studies particularly memorable.

Finally, I am indebted to The Wellcome Trust for funding this study (035589/1.5B).

ABSTRACT

The distribution of the granule chymase Sheep Mast Cell Proteinase (SMCP) was determined in trachea, bronchus, bronchial lymph node, lung, thymus, spleen, liver, flank skin, abomasum, duodenum, jejunum, ileum, colon and mesenteric lymph node by immunohistochemistry and by ELISA using a polyclonal, affinity purified anti-SMCP antibody. The toluidine blue and SMCP-positive cell counts were closely correlated for all tissues examined ($r^2 = 0.96$, $P<0.001$), with the exception of skin and liver. On the basis of reactivity to the anti-SMCP antibody, two populations of ovine mast cells were identified. SMCP-positive cells (analogous to the gastrointestinal or mucosal mast cell [MMC] subset) were present in all tissues examined whereas SMCP-negative cells were present in skin (the putative ovine connective tissue mast cell [CTMC] subset) and comprised ~98% of the ovine dermal mast cell population. The functional heterogeneity of the ovine dermal mast cell population was investigated in cutaneous challenge studies using the secretagogues calcium ionophore A23187 (A23187), substance P (sP) and compound 48/80 (48/80), which are known to activate CTMC subsets in other species. Although only A23187 and sP evoked an immediate weal response ($P<0.05$; Mann-Whitney U test [MW]), all three agents evoked dermal neutrophil influx ($P<0.05$; MW) with extensive mast cell degranulation ($P<0.05$; MW), thus identifying these agents as putative ovine dermal mast cell secretagogues. As SMCP may be released into the dermis following degranulation, its effect in ovine skin *in vivo* was investigated. SMCP (36µg - 36ng/50µl) evoked a dose-dependent immediate cutaneous response characterized by weal formation (maximal by three hours after injection ($P<0.05$; MW)) accompanied by dermal neutrophil influx ($P<0.05$; MW) and concomitant mast cell degranulation ($P<0.05$; MW). There was no subsequent delayed component to this response (24 to 72 hours). Although heat-inactivation of SMCP (64°C for 10 min; ~2% residual

activity) abrogated the weal response ($P<0.05$-$P<0.01$; MW), there was no effect on dermal neutrophil influx. Recombinant ovine interleukin-3 (rOv.IL-3) was shown to consistently generate a population of rOv.IL-3-dependent bone marrow-derived mast cells (rOv.IL-3 BMMC) *in vitro*, this cell population being subsequently used to compare functional heterogeneity *in vitro* to that previously determined in skin *in vivo*. These generated cells contained the granule-associated mediators arylsulfatase, β-hexosaminidase and SMCP, the latter finding being consistent with an MMC phenotype. A dose-dependent effect of rOv.IL-3 on cell viability and the maximum percentage of SMCP-positive mast cells obtained was observed ($P<0.05$-$P<0.01$; Student's *t*-test), the latter being increased by transferring the non-adherent cell population to fresh wells or flasks at feeding. When harvested optimally at days 12 to 16 of culture, these rOv.IL-3 BMMC could be activated by sP, 48/80 and A23187 to release arylsulfatase, β-hexosaminidase and SMCP, indicating that these cells may also possess CTMC characteristics. Thus, r.Ov.IL-3 BMMC may represent a cell population of mixed (MMC and CTMC) phenotype. SMCP failed to evoke similar mediator release *in vitro*, in contrast to the immediate cutaneous response observed *in vivo*. One action of SMCP may therefore be to activate vascular endothelium, thereby promoting increased vascular permeability and subsequent dermal neutrophil influx.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A23187	calcium ionophore A23187
β-hex.	β-hexosaminidase
BMMC	bone marrow-derived mast cell(s)
BSA	bovine serum albumin
CM	conditioned medium
CTMC	connective tissue mast cell(s)
DAB	3,3′-diaminobenzidine
DFP	diiosopropyl fluorophosphate
ELISA	Enzyme-linked immunosorbent assay
48/80	compound 48/80
FCS	foetal calf serum
FPLC	fast protein liquid chromatography
Ig	immunoglobulin
IL	interleukin
i.d.	intradermal
HBSS	Hanks' Balanced Salt Solution
HI-SMCP	heat-inactivated sheep mast cell proteinase

IMDM	Iscove's modification of Dulbecco's medium
kDa	kilodaltons
LT	leukotriene
µg	micrograms
mg	milligrams
MMC	mucosal mast cell(s)
MMCP	mouse mast cell proteinase
MW	molecular weight
ng	nanograms
NGS	normal goat serum
NRS	normal rabbit serum
O.D.	optical density
Ov.	ovine
PBS	phosphate-buffered saline
PG	prostaglandin
rabαSMCP	rabbit anti-SMCP
RMCP	rat mast cell proteinase
ratαSMCP	rat anti-SMCP
rOv.IL-3	recombinant ovine interleukin-3

r.p.m.	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
s.c.	subcutaneous
SEM	standard error of the mean
SMCP	sheep mast cell proteinase
SP	substance P
$^{w}/_{v}$	weight in volume

CHAPTER 1

GENERAL INTRODUCTION

1.1. The Mast Cell And Cutaneous Inflammation

Inflammation can be defined as the "cellular and vascular responses of living tissue to injury" (Thompson 1978). The mast cell, as one of the participating cells in the skin immune system (Bos and Kapsenberg 1986), contributes to the development and modulation of cutaneous inflammation following activation (Walsh, Lavker and Murphy 1990, Harvima et al 1994) as a consequence of the synthesis and release of preformed or newly synthesized mediators and cytokines (reviewed in Galli 1993, Marshall and Bienenstock 1994, Schwartz 1994). Mast cell activation classically occurs when IgE (bound by its Fc portion to FcεRI) recognises specific mutivalent antigen (Galli 1993), but other agents such as neuropeptides (Foreman 1993) and components of the complement cascade (Füreder et al 1995a) can also evoke dermal mast cell degranulation (reviewed in Amon et al 1994). Additionally, a potential role for the mast cell in persistent or chronic inflammatory responses, rather than merely the classical acute or anaphylactic-type response (e.g. urticaria), has been advocated (Askenase 1977, Dvorak et al 1976, Galli 1993, Torii et al 1993). Cutaneous mast cells differ from mast cells at other tissue sites with respect to a range of important characteristics (e.g. functional activity, mediator content, proteinase content; Barrett and Pearce 1993) which can influence the nature of the inflammatory response in skin compared to other tissues, and which also defines the heterogeneity of the dermal mast cell population in various species.

Mast cells are believed to contribute to, or be associated with, various dermatoses in both man and domestic animals (Table 1.1). In routine small animal veterinary practice, about 25% of all cases seen are dermatological in nature (Grant and Thoday 1991), of which canine atopic disease alone accounts for between 2% and 8% of all skin cases (Halliwell and Schwartzman 1971, Scott 1981). In sheep, apart from the detrimental effect on health and general welfare, substantial economic losses can occur due to skin disease. For example, in a single flock outbreak of *Psoroptes ovis* infestation ("sheep scab"), the reduction in weight gain alone was

Veterinary Dermatoses	Evidence cited for mast cell involvement
Canine Atopic Disease	Halliwell, Schwartzman and Rockey 1972, Nimmo-Wilkie et al 1990
Canine Flea-Bite Hypersensitivity	Kieffer and Kristensen 1979; Gross and Halliwell 1985; Halliwell and Longino 1985; Gross, Ihrke and Walder 1992;
Canine demodicosis	Copeman 1965
Feline *Otodectes cynotis* infestation	Weisbroth et al 1974; Powell et al 1980
Mast cell neoplasia	Bostock 1973, 1986
Ovine cutaneous atopic disease	Scott and Campbell 1987
Ovine *Culicoides* hypersensitivity	Connan and Lloyd 1988
Ovine fleece rot and fly strike (*Lucilia cuprina*)	Colditz et al 1994
Ovine Orf virus infection	M^{c}Ewan Jenkinson, Hutchison and Reid 1992
Human Dermatoses	**Evidence cited for mast cell involvement**
Urtica dioica urticaria	Oliver et al 1991
Physical urticaria	Hawk et al 1980, Kobza-Black et al 1979
Chronic idiopathic urticaria	Elias, Boss and Kaplan 1986; Kaufman and Rosenstreich 1990; Smith, Soh and Lee 1992; Jacques et al 1992
Mastocytosis	Olafsson, Roupe and Enerbäck 1986; Irani et al 1990
Scleroderma and fibrosis	Nishioka et al 1987; Takeda, Hatamochi and Ueki 1989; Claman 1989
Acute sunburn response	Walsh 1995

Table 1.1. Selected dermatoses in domestic animals and man where an association with mast cells has been reported.

equivalent to a £1000 loss (Kirkwood 1980). In addition to a wide range of dermatoses caused by ectoparasites (e.g. Chorioptic and Psoroptic mange, tick infestation, pediculosis), bacterial (e.g. Staphylococcal folliculitis), fungal (e.g. mycotic dermatitis) and viral (e.g. Orf virus infection) dermatoses are common in sheep (reviewed in Martin and Aitken 1991). Mast cells have been postulated to play a role in immediate hypersensitivty responses to ectoparasite infestation (Scott 1988), and may also contribute to other ovine hypersensitivity disorders, given that cutaneous atopic disease has also been demonstrated in the sheep (Scott 1988). Thus, it is of importance that the contribution of the mast cell to ovine cutaneous inflammatory responses is elucidated.

Therefore, the focus of this review is the cutaneous mast cell, its heterogeneity and its possible role in the development and modulation of cutaneous inflammatory responses with particular emphasis on the mast cell proteinases, tryptase and chymase.

1.2. Historical Aspects Of Cutaneous Mast Cells

Von Recklinghausen (1863) first identified the presence of granular cells at connective tissue sites during studies on frog mesentery. Using a differential staining technique, Ehrlich (1877) considered these cells to be a type of "plasma cell". These cells were further characterized as metachromatic connective tissue cells, and the term "Mastzellen" was coined (Ehrlich 1878, 1879). A role for mast cells in cutaneous pathology was advocated by Unna (1887, 1894), when increased numbers of mast cells were noted in various dermatoses, including urticaria pigmentosa. Mast cells have subsequently been demonstrated in the skin of man (Staemmlar 1921, Vallone 1944, Hellström and Holmgren 1950, Eady et al 1979), horse (Dozza and Rampichini 1963, Talukdar, Calhoun and Stinson 1972), cow (Riley 1959, Jenkinson et al 1970), pig (Riley 1959), sheep (Kozlowski and Calhoun 1969), goat (Scott, Smith and Manning 1984), dog (Riley and West 1956, Emerson and Cross 1965), cat (Riley and West

1956), rat (Iakovleva 1954), opossum (Santos and Machado 1994), guinea pig (Zimmermann 1908), hamster (Compton 1952), mouse (De Viñals 1931) and also in fish scales (Veil 1957).

Cutaneous mast cells are located in the dermis in most species, in close association with blood vessels, pilosebaceous units, arrector pili muscles and unmyelinated nerves [i.e. in perivascular or periadnexal locations](Kozlowski and Calhoun 1969, Van Loeveren, Teppema and Askenase 1990, Scott, Miller and Griffin 1995). However, both normal (Zelickson 1985) and diseased (Green, Cordero and Winklemann 1977) human skin is said to contain epidermal mast cells. Similarly, in a study of various non-neoplastic feline dermatoses (Scott 1990), 11.6% of cases (37 of 338 cats) possessed epidermal mast cells. No epidermal mast cells have been demonstrated in studies of ovine skin, mast cells being associated predominantly with wool follicles, capillaries, sweat glands, sebaceous glands and arrector pili muscles in the dermis (Kozlawski and Calhoun 1969, Vegad 1970). As in other species (e.g. man, Brix 1963; dog, Emerson and Cross 1965) mast cell numbers in sheep skin vary with the anatomical site, increased numbers of mast cells being detected in one study in periorbital, interdigital and inguinal areas (Kozlowski and Calhoun 1969). Variations in the vascular response to a standard injection of histamine in sheep was apparently dependent on whether it was administered into dorsal or ventral flank skin (Colditz 1988). Care must therefore be taken when selecting a site on which to perform cutaneous response studies, as such variation may influence results. This is particularly relevant in studies where large areas of ovine flank skin are used to assess responses (e.g. Colditz 1988, Yilmaz, Haverson and Morgan 1994). However, where relatively small areas of flank are used (as in Chapters 4 and 5), this effect is considered unlikely to affect results (Colditz 1995, *personal communication*).

1.3. The Origin Of Mast Cells

Mast cells are derived from precursors which originate in bone marrow (Kitamura et al 1977) and migrate via the bloodstream (Kitamura et al 1979, Sonoda, Ohno and Kitamura 1982) to complete their differentiation in peripheral sites where their final phenotype is determined (Kitamura, Matsuda and Hatanaka 1979, Huff et al 1995). However, whether the majority of mast cell-committed progenitors (unipotential ungranulated colony forming units) reside in bone marrow or in peripheral tissues is still debatable (Huff et al 1995). Mast cell development contrasts markedly with that of the basophil, which undergoes its terminal differentiation in the bone marrow and is subsequently released into the bloodstream as a mature cell (reviewed in Galli 1990).

1.4. Mast Cells And T Lymphocyte Interactions - Relevance To Mast Cell Heterogeneity And To The Development Of Hypersensitivity Responses

The T cell dependency of the mucosal mast cell (MMC) population was defined in rodents. Adoptive transfer studies with T cell-enriched thoracic duct lymphocytes from primed donor rats results in intestinal mastocytosis in the recipients (Nawa and Miller 1979). Congenitally athymic (nude) mice do not demonstrate MMC hyperplasia in response to *Trichinella spiralis* infection (Ruitenberg and Elgersma 1976) and rats depleted of T cells show a similar lack of response to *Nippostrongylus brasiliensis* infection (Mayrhofer 1979, Mayrhofer and Fisher 1979). Also, supernatants from activated T cells (conditioned medium; CM) can support the growth of rat (Haig et al 1982, Haig et al 1983) and mouse (Schrader et al 1981) bone marrow-derived mast cells (BMMC). Unlike the MMC population, the connective tissue mast cell (CTMC) populations in mouse and rat are not considered to be T cell-dependent (Katz, Stevens and Austen 1985, Stevens and Austen 1989, Galli 1990).

Two types of murine $CD4^+$ T helper cell clones (T_{H1} and T_{H2}) were defined, based on their cytokine-producing phenotype (Mosmann et al 1986, reviewed in Arai et al 1990, Paul and

Seder 1994), this subsequently being extended to include normal human T cell populations (Del Prete et al 1991). When activated, both T_{H1} and T_{H2} cells produce interleukin-3 (IL-3), granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α). However, additionally, T_{H1} cells produce IL-2, interferon-γ (IFNγ) and TNF-β to enhance cellular immunity, particularly with regard to increased macrophage-induced IL-12 production (Paul and Seder 1994). T_{H2} cells, upon activation, produce IL-4, IL-5, IL-6, IL-9, IL-10 (Mosmann et al 1991) and IL-13 (Paul and Seder 1994), which (particularly IL-4) acts to help B cell development to produce antibody-producing cells. IL-4 acts as a co-stimulant with CD40 ligand (Banchereau et al 1991) on B cell growth, and controls immunoglobulin class switching of B cells expressing IgM to two isotypes, IgG_1 and IgE in mouse (Finkelman et al 1986) and man (in conjunction with IL-13; Punnonen et al 1993).

Cytokine cross-regulation of priming by the T_{H1} and T_{H2} subsets occurs (Paul and Seder 1994). Thus, T_{H2}-generated IL-4 inhibits the IL-12 (derived from activated macrophages) priming of precursor T_H (pT_H) cells for IFNγ production and subsequent development of a T_{H1} phenotype (Seder et al 1992, Seder et al 1993). Likewise, IFNγ inhibits the IL-4 priming of pT_H cells for subsequent generation of a T_{H2} phenotype (Seder et al 1992). T_{H2} cells themselves need an exogenous pulse of IL-4 to initiate their differentiation and synthesis of IL-4. Mast cells generate several cytokines upon immunological or non-immunological activation (Burd et al 1989, reviewed in Gordon, Burd and Galli 1990; discussed below), including IL-3, IL-4, IL-5 and IL-6, thereby resembling the secretion pattern of T_{H2} cells (Plaut et al 1989). Importantly, human cutaneous (Bradding et al 1992) and bronchial/nasal (Bradding et al 1992, 1993) mast cells are a source of IL-4. Additionally, IL-4 is also detectable in mast cells from lesional and non-lesional skin in human atopic dermatitis (Horsmanheimo et al 1994). Human lung mast cells also express CD40-ligand, and have been

shown *in vitro* to stimulate B cells directly to switch to IgE antibody production (Gauchat et al 1993). This, in conjunction with mast cell-derived IL-4 and IL-6 (which augments antibody production, including IgE, by B cells; Vercelli et al 1989), could encourage IgE production and the development of an atopic state. Thus, mast cells and T cells are closely inter-related in the initiation and development of immunological responses.

1.5. Proteinases

1.5.1 Serine Proteinases - General Introduction

Proteinases (or proteases; proteolytic enzymes) can be subdivided into exopeptidases (which cleave bonds near to the ends of polypeptide chains) and endopeptidases (which cleave bonds distant from the ends of polypeptide chains). Endoproteinases have been classified into four groups (Hartley 1960) dependent on the nature of the catalytic site of the enzyme into serine-, cysteine-, aspartic- and metalloproteinases. Serine proteinases, of which mast cell tryptases and chymases are members, contain a serine residue at the catalytic site and contain a characteristic "catalytic triad" of histidine, aspartate and serine residues (Springman and Serafin 1995). The serine residue (in chymotrypsin) sits in a deep cleft which accounts for its specificity for aromatic and other bulky hydrophobic side chains. Classically, the serine proteinases can be inhibited by diisopropyl fluorophosphate (DFP).

1.5.2. Mast Cell Tryptases And Chymases

Gomori (1953) first demonstrated that mast cells were the site of unusual hydrolytic enzymatic activity. Mast cell-derived enzymes were later shown to possess both chymotryptic (Benditt and Arase 1959, Lagunoff and Benditt 1961, Lagunoff and Benditt 1963) and tryptic-like activities (Glenner and Cohen 1960, Lagunoff and Benditt 1963), the terms chymase (originally "chymotrypsin-like esterase of mast cells; Benditt and Arase 1959, Glenner and Cohen 1960, Lagunoff and Benditt 1963) and tryptase (Lagunoff and Benditt 1963) being introduced to identify these respective enzymes. Mast cell proteinases have been

extensively reviewed (Nadel 1991, Walls 1995) with a number of them concentrating on the role of proteinases in skin and in skin disease (Harvima et al 1994, 1995; Schechter 1995). Primate and canine mast cells contain large amounts of tryptase, in comparison to mast cells of the rat (which have little tryptase except in the lung; Chen et al 1993) and rabbit which contains no tryptase (Chiu and Lagunoff 1972). Although guinea pig mast cells were also originally considered not to contain tryptase (Chiu and Lagunoff 1972), recently tryptase has been identified in mast cells from this species (He et al 1995). In the mouse, although two tryptases are present (MMCP-6 and -7; Stevens et al 1994), these are not expressed by gastrointestinal MMC (Reynolds, Austen and Serafin 1992) but can be found in CTMC (Stevens et al 1994). Therefore, chymase is the predominant enzyme in rodent mast cells, which contrasts with the dog and man.

Tryptase exists in an active form in mast cells (Lagunoff, Benditt and Watts 1962, Lagunoff and Benditt 1963), a species-specific trypsin-like enzyme having been initially detected by histochemical methods (Glenner and Cohen 1960), and accounts for between 20% and 50% of the entire protein content of mast cells (Harvima and Schwartz 1993). Tryptase has been purified from human lung (Schwartz, Lewis and Austen 1981) and from skin (Harvima et al 1988). Tryptase is a tetrameric endoproteinase of 134kDa, with subunits of 31kDa to 34kDa (Schwartz 1995). Tryptase, within mast cell granules and after secretion, is associated with heparin (Caughey et al 1988a), the ionic binding of tryptase to heparin (under physiological conditions) through a histidine rich sequence (demonstrated with MMCP-7; Matsumoto et al 1995) preventing its dissociation into inactive monomers (Alter et al 1987). Chondroitin sulphate E can also stabilise tryptase to some extent (Schwartz 1995). (It should be noted that, in contrast to human tryptase, rat tryptase does not bind heparin (Lagunoff, Rickard and Marquardt 1991)). Tryptases possess acidic pI values (e.g. MMCP-6 6.50, MMCP-7 5.95) which contrasts markedly with the basic pI values of chymases (e.g. MMCP-4 9.66), this

being of relevance to the proteoglycan binding characteristics of tryptase and chymase (Springman and Serafin 1995). Post-translational modification of proteinases, although not essential for proteinase function (e.g. Rat Mast Cell Proteinase-I [RMCP-I] and RMCP-II are non-glycosylated), can account for observed differences in proteinase stability, solubility, proteoglycan binding/packaging and activity. For example, in the mouse, the activity of MMCP-1 is dependant upon the particular glycoform (A to E) that is present (Newlands et al 1993). Tryptase hydrolyses proteins on the C-terminal side of the basic/positively charged residues lysine and arginine (Schwartz 1985) and favours cleavage of tripeptide substrates with basic amino acids in the S1 and S2 positions (Schwartz 1995; biological substrates for tryptase will be discussed below). Two tryptase cDNA molecules have also been cloned from a human lung mast cell cDNA library and designated as α-tryptase (Miller, Westin and Schwartz 1989) and β-tryptase (Miller, Moxley and Schwartz 1990). Three cDNA molecules (I, II and III) were also cloned from a human skin mast cell library, the β-tryptase cDNA amino acid product being 98% to 100% homologous with that from tryptase I, II and III cDNA (Vanderslice et al 1990). Tryptase is not inhibited by classical serine proteinase inhibitors (Harvima et al 1988, Alter et al 1990, Schwartz 1995) such as lima bean, soybean or ovomucoid trypsin inhibitors which distinguishes it from other serine proteinases, although it can be inhibited by low molecular weight substances such as leupeptin, DFP and phenylmethylsulfonyl fluoride and is also rapidly inactivated in low salt conditions (Schwartz 1995). Presently, no specific physiological inhibitor has been found for tryptase (Harvima et al 1995). Loss of tryptase activity may result from destabilization of the tryptase-heparin complex *in vitro* by heparinases (Alter et al 1987) or by heparin binding proteins (Alter et al 1990) in conjunction with divalent cations (Alter and Schwartz 1989). However, the mechanism by which tryptase is dissociated from heparin *in vivo* is unknown (Schwartz et al 1995). Certainly, it is stated that active tryptase has a prolonged action following release

from mast cells in skin, in part due to its interaction with heparin, although no figures are quoted for the duration of its action (Harvima et al 1995). Given that the proteoglycan content of mast cell subpopulations differs between individual anatomical locations (discussed below), then the nature of the proteoglycan could affect the relative biological activity of tryptase in different tissues. The possible contribution of heparin to the biological regulation of proteinase activity should also be addressed when considering experiments in which purified proteinases are injected *in vivo*. For example, the intradermal injection of tryptase plus heparin in ovine skin produces an immediate cutaneous response of greater magnitude than that due to tryptase alone (Molinari et al *in press*). This could reflect less rapid inactivation via destabilization of tryptase-bound heparin and/or persistence at the site of injection due to the relative size of the tryptase-heparin complex.

Human chymase has been purified from skin (Schechter et al 1983), lung (Wintroub et al 1986) and heart (Urata et al 1990). Chymase is a monomeric serine proteinase of 30kDa by SDS-PAGE (Wintroub et al 1986) although murine chymases (MMCP-1 to MMCP-5) range between 26kDa and 36kDa (Springman and Serafin 1995). The gene for human skin chymase has been cloned (Caughey, Zerwick and Vanderslice 1991). As mentioned above, chymases possess basic pI values and the characteristic catalytic triad of histidine, aspartate and serine residues, its endopeptidase activity being measured by cleavage of synthetic p-nitroanilide-peptide conjugates (Harvima and Schwartz 1993). The strong positive charge associated with chymase allows it to bind to other cells and to basement membranes following dissociation from granule proteoglycans (discussed below; Walls 1995). Cleavage of a propeptide in the initially translated proenzyme allows spontaneous refolding to produce the mature, active site (Springman and Serafin 1995). Chymotrypsin-like proteinases cleave at large hydrophobic residues such as tryptophan, tyrosine, phenylalanine, leucine and methionine. For example, chymases in man, dog and rodents demonstrate an extended substrate binding site with a

preference for phenylalanine at residue P_1 and for hydrophobic residues at P_2 and P_3 (Powers et al 1985). Chymase is stored in an active form in mast cells (Huntley et al 1985) in conjunction with heparin (Harvima and Schwartz 1993). Indeed, chymase and tryptase are stored in the same mast cell granule (Craig, Schechter and Schwartz 1988), although chymase is released in a complex along with carboxypeptidase separate from tryptase (Goldstein et al 1992). The chymase complex (with proteoglycan and carboxypeptidase) is between 400kDa to 560 kDA (tryptase complex is between 200kDa and 250kDa; Walls 1995) and thus there is a greater relative possibility of chymase retention in comparison to tryptase at sites of release. This could directly affect the degree of cutaneous response with regard to release of the two proteinases. However, unlike tryptase, human skin chymase remains active in the absence of heparin under physiological conditions (Sayama et al 1987). In the same study it was shown that heparin binding did not affect the cleavage of small peptide substrates but it was speculated that such binding could reduce the accessibility of the active site to proteins (Sayama et al 1987). This is supported by data that indicates that only free RMCP-I (i.e. not granule associated) can hydrolyze larger protein molecules (Le Trong, Neurath and Woodbury 1987). The binding of chymase to heparin within mast cell granules does not block the active site of the proteinase (Sayama et al 1987) and indeed enhances the activity of chymase by 33% at pH 7.5 (the pH of the extracellular space), in comparison to a depression in activity of 40% at pH 5.5 (pH of the mast cell granule) (M^{c}Euen, Sharma and Walls 1995). Significantly, the presence of heparin was shown to prevent inhibition of RMCP-I activity by the serpins (serine proteinase inhibitors) α_1-proteinase inhibitor (α_1-PI) and α_1-antichymotrypsin, and by α_2-macroglobulin, soybean trypsin inhibitor and plasma (Pejler and Berg 1995). Additionally, dissociation of RMCP-I from heparin was shown to result in a reduced ability of the chymase to inactivate thrombin (Pejler, Soderstrom and Karlstrom 1994). Thus, based on the *in vitro* evidence above, the glycosaminoglycan-proteinase

interactions can be speculated to be of functional significance and not merely as a mechanism to concentrate intracellular molecules (discussed in Sayama et al 1987).

Chymase is inhibited by the classical inhibitors of serine proteinases, including DFP (Harvima and Schwartz 1993). The interaction of chymase with low molecular weight inhibitors is reviewed by Schechter (1995). Chymase can be inhibited by serpins such as α_1-antichymotrypsin and α_1-PI (Schechter et al 1989) but these act as better substrates than inhibitors for chymase, this therefore implying that plasma is not solely responsible for regulation of chymase activity (Schechter et al 1989). Of particular interest to the regulation of chymase in the skin is the presence of the proteinase inhibitor Elafin in psoriatic epidermis (Wiedow et al 1990) and that α_1-PI and α_1-antichymotrypsin have been detected in human skin mast cells (Ruck, Horny and Kaiserling 1990, Harvima et al 1993). Certainly, chymase can be inhibited by the serpins α_1-antichymotrypsin and α_1-PI in plasma ($k_{ass} \approx 7.5 \times 10^3\ M^{-1}s^{-1}$ for human chymase and α_1-PI), as well as partially by α_2-macroglobulin (Schechter et al 1989). The half-life for chymase (based on the combined inactivations of chymase by α_1-PI and α_1-antichymotrypsin) was calculated to be 1.3s, this being 650 times slower than for inactivation of neutrophil cathepsin G in the same study (Schechter et al 1989). This, in conjunction with the estimated dermal concentration of chymase (after complete mast cell-release) of 1μM, suggests that chymase has the potential to contribute substantially to proteolytic activity in the dermis.

1.5.3. Sheep Mast Cell Proteinase (SMCP)

When mucosal mast cells (MMC) were isolated from the abomasal mucosa of *Ostertagia circumcincta* parasitized sheep (Huntley, Wallace and Miller 1982), a chymotrypsin-like esterase was demonstrated histochemically within these abomasal mucosal mast cells (Huntley, Newlands and Miller 1984, Huntley et al 1985). A cationic proteinase, sheep mast

cell proteinase (SMCP) was successfully isolated from abomasal mucosal mast cells (Huntley et al 1986), and the mast cell source of this enzyme confirmed immunohistochemically (Huntley et al 1986). The catalytic properties of SMCP were initially defined as those of a chymotrypsin-like enzyme (Knox, Gibson and Huntley 1986), this description being refined to that of a serine endopeptidase (Knox and Huntley 1987). Following development of an ELISA to detect SMCP, it was demonstrated that SMCP was released locally into lymph and systemically into blood following abomasal challenge with *Haemonchus contortus* and *Ostertagia circumcincta* respectively (Huntley et al 1987), despite the presence of inhibitors of the ELISA in both lymph and serum (Huntley et al 1987, Huntley 1991). Increased blood pepsinogen activity (Smith et al 1984) accompanied the raised SMCP levels demonstrable following challenge with *Ostertagia circumcincta* (Huntley et al 1987; the same samples being analysed as in Smith et al 1984), suggesting that the former was a consequence of a hypersensitivity response involving MMC. SMCP hydrolyses substance P (sP), bradykinin, met-lys-bradykinin and oxidised insulin B chain at P_1 Phe or Leu residues in accordance with its chymotrypsin-like activity (Pemberton, Huntley and Miller, *submitted*). However, SMCP exhibits an unexpected trypsin-like activity on bovine, ovine and equine serum albumin, cleaving bovine serum albumin after P_1 Lys residues flanked by acidic amino acids at the P_4 and P_4' positions (Pemberton, Huntley and Miller, submitted). Following purification from sheep serum, α_1-PI and α_2-macroglobulin were confirmed as the two most potent inhibitors of SMCP present therein (Pemberton, Huntley and Miller, submitted). Like human chymase (Schechter et al 1989), SMCP is inhibited relatively slowly (≈15 minutes) by its homologous α_1-proteinase inhibitor ($k_{ass} \approx 1.1 \times 10^3$ $M^{-1}s^{-1}$; Pemberton, Huntley and Miller, *submitted*), which would enable its persistence at the site of cutaneous responses but also enable it to be functional at some distance from its point of release (or injection).

A second putative chymase (in addition to SMCP) was detected by Western blotting in ovine bone marrow cells grown in the presence of lymphocyte-derived conditioned media from nematode-infected sheep (CM-BMMC) but it was not present in blots from purified gastric MMC (Huntley et al 1992). Additionally, mast cell tryptase has yet to be isolated from ovine tissues, although tryptase has been isolated from bovine liver capsule (Fiorucci, Erba and Ascoli 1992). However, it has been shown that APC 366 (a tryptase inhibitor) reduces both the late phase response evoked by allergen challenge (Clark et al 1995) and the mast cell-mediated immediate cutaneous response to intradermally injected *A.suum* antigen in allergic sheep (Molinari et al, *in press*). These results suggest that tryptase may be present in ovine mast cells.

Thus, even from the broad, largely *in vitro*, biochemical data outlined (1.5.1.-1.5.3.) above there is evidence that chymases and tryptases possess marked proteolytic potential and to be relatively persistent once released from activated mast cells. The hypothesis that they are able to participate in cutaneous inflammatory responses will be expanded upon by consideration of their potential biological substrates, discussed later.

1.6. Mast Cell Heterogeneity

1.6.1. General Introduction And Contribution Of Mast Cell Proteinases

Currently, comparatively little is known about the ovine mast cell nor of mast cell phenotypic heterogeneity in the sheep. As phenotypic heterogeneity is one theme of the studies described in this thesis, a brief review will be given of this topic. Indeed, mast cells in various species are a phenotypically heterogeneous cell population with respect to a range of characteristics (reviewed in Bienenstock 1988, Galli 1990, Huntley 1992, Barrett and Pearce 1993). These characteristics can include tissue site, fixation characteristics, and histochemical staining patterns (Becker et al 1985, Aldenborg and Enerbäck 1988), biochemistry (Katz, Stevens and

Austen 1985, Stevens et al 1986a), proteinase content (Gibson and Miller 1986, Irani et al 1986, Reynolds et al 1990), cytokine content (Bradding et al 1995) and functional activity (Foreman 1993, Amon 1994).

Enerbäck (1966a, 1966b) demonstrated histochemical differences between rat dermal mast cells (CTMC) and rat intestinal MMC, cytoplasmic granules staining red and blue respectively following sequential treatment with alcian blue and safranin. This distinction is not absolute as there is variability in safranin staining at different connective tissue-type sites in the rat (Aldenborg and Enerbäck 1988, Tainsh and Pearce 1992). Additionally, differences in tissue fixation (formalin sensitivity) and staining exist within the canine dermal mast cell population (Becker et al 1985). Similarly, within both human intestine (Befus et al 1985) and skin (Marshall, Ford and Bell 1987) formalin-sensitive and -insensitive sub-populations exist. Tissue fixation and histochemical staining of glycosaminoglycans are therefore inconsistent, proving an unreliable guide to the delineation of mast cell subsets.

Analysis of the content of mast cell-specific neutral proteinases is currently the most definitive method of comparing heterogeneity between mast cell populations (Miller et al 1989, Galli 1990, Miller 1993a). Mast cell heterogeneity is based upon either the presence or absence of chymase in addition to tryptase (as in man) or on differences in the types of chymases expressed (as in rodents), and is broadly outlined in Table1.2. and briefly discussed below.

	DISTRIBUTION OF SERINE PROTEINASES IN THE GRANULES OF MAST CELLS FROM DIFFERENT TISSUES			
Mast Cell Population	**Rat**	**Mouse**	**Man**	**Sheep**
Cutaneous	RMCP-I (Tryptase)	(MMCP-2) MMCP-4 MMCP-5 MMCP-6 (MMCP-7)	Tryptase Chymase	?
Intestinal	RMCP-II	MMCP-1 MMCP-2	Tryptase (Chymase)	SMCP
Pulmonary (Parenchyma)	RMCP-I and II + Tryptase	?	Tryptase	?
Pulmonary (Large Airways)	RMCP-II	?	Tryptase Chymase	?

Table 1.2. General distribution of serine proteinases in the granules of mast cells from different tissues. A tissue in which the exact distribution is presently unknown is delineated by "?".

1.6.1.1. Rat

Following the initial isolation of mast cell chymases in the rat (Lagunoff and Benditt 1963, Lagunoff and Pritzl 1976, Yurt and Austen 1977, Woodbury et al 1978), two distinct chymases were identified, antibodies to these being used to define mast cell heterogeneity (Gibson and Miller 1986, Gibson et al 1987). Rat mast cell proteinase-I (RMCP-I) is associated with the CTMC subset (i.e. skin), whereas rat mast cell proteinase-II (RMCP-II) is predominantly found in the gastrointestinal MMC subset (Gibson and Miller 1986, Gibson et al 1987). However, RMCP-II-positive mast cells have been demonstrated at non-mucosal sites (e.g. thymus; Gibson and Miller 1986) and, additionally, dual RMCP-I- and RMCP-II-staining cells have been identified at tissue sites including the gastric submucosa and mesenteric lymph node (Huntley et al 1990). Thus, although general proteinase heterogeneity exists in the rat, proteinase heterogeneity also occurs within certain rat mast cell populations. Tissue location alone cannot therefore be used to explicitly define all mast cell subsets in the rat, although no RMCP-II has as yet been demonstrated in skin (Chernin et al 1988), indicating that RMCP-I can be used to define dermal mast cells in this species.

1.6.1.2. Mouse

Seven mouse mast cell proteinases (MMCP) have currently been identified, these including five chymases (MMCP-1 to MMCP-5) and two tryptases (MMCP-6 and MMCP-7) (Newlands et al 1987, Reynolds et al 1990, Schwartz 1994), although MMCP-7 has presently only been detected as the mRNA in interleukin-3-dependent BMMC (IL-3 BMMC) *in vitro* (M^{c}Neil et al 1992). (N.B. MMCP-L represents a further putative proteinase identified when a mouse genomic library was screened with an MMCP-4 cDNA probe [Springman and Serafin 1995]. However, this has not been shown to be present in any mast cell type as yet). MMCP-1 is present in the MMC subset (Newlands et al 1987, Le Trong et al 1989), and is not demonstrable in any connective tissue mast cell subset (Newlands et al 1993, Miller et al

1995). MMCP-3, MMCP-4 and MMCP-5 also help delineate the CTMC subset (Stevens et al 1994, Schwartz 1994). However, MMCP-2 (which is considered a predominantly MMC proteinase) can also be detected immunohistochemically in murine cutaneous mast cells (Stevens et al 1994). Thus, as MMCP-1 is absent in skin, tongue or peritoneal mast cells (Newlands et al 1993, Miller et al 1995), then this provides a correlation between proteinase content and mast cell subtype (MMC or CTMC) in the mouse.

1.6.1.3. Man

Mast cell heterogeneity in man is defined by the presence or absence of chymase in cells which all contain tryptase. This has been used to delineate two mast cell subtypes, the MC^{TC} (containing both tryptase and chymase) and MC^{T} (containing tryptase alone) (Irani et al 1986, Schwartz 1989, Irani and Schwartz 1990). The MC^{TC} is chiefly present in skin and small intestinal submucosa, whereas MC^{T} is present in lung and small intestinal mucosa (Irani et al 1986, Schwartz et al 1987, Schwartz 1989, Irani and Schwartz 1990). The MC^{TC} subtype accounts for between 88% and 99% of mast cells in skin (Irani et al 1986, Schwartz et al 1987). In addition, a third subtype of mast cells containing chymase alone (MC^{C}) has been described, although these cells are present in extremely low numbers in skin (Weidner and Austen 1993).

1.6.1.4. Dog

Most work on canine proteinases has focused on their isolation or release from mastocytoma cell lines (Slavin et al 1987, Caughey et al 1988a, Caughey et al 1988b, Schechter et al 1988) or on their biological activity (reviewed in Sommerhoff 1995). Canine dermal mast cells contain both a chymase and a tryptase (Schechter et al 1988), these proteinases being differentially expressed in mastocytoma cell lines (Caughey et al 1988c). Additionally, a second, tryptase-related proteinase (Dog Mast Cell Proteinase-3, dMCP-3) has recently been identified, and demonstrated immunohistochemically (using an antibody raised against a

peptide [residues 166-181] from the deduced sequence) in mast cells of the lung, jejunum and dermis (Yezzi, Hsieh and Caughey 1994). However, dMCP-3 can also be detected in canine neutrophils (Yezzi, Hsieh and Caughey 1994). In one study which looked at the distribution of tryptase in the dog (Myles et al 1995), the gastrointestinal tract was shown to have the highest tryptase levels with lower levels in skin (30ng/mg [dorsum] to 68ng/mg [ventrum]). However, further morphometric analysis is required before mast cell proteinase heterogeneity can be defined in the dog, and is therefore not included in Table 1.2.

1.6.1.5. Sheep

Although the concentration of SMCP in the gastrointestinal mucosa is known to be significantly correlated with mast cell counts (Huntley 1991), the distribution of SMCP in non-mucosal tissues was unknown (Table 1.2). Thus, it was also unknown whether heterogeneity, as demonstrated in other species based on proteinase content, existed in the sheep. This, therefore, provided the impetus to elucidate the tissue distribution of SMCP.

1.6.2. Proteoglycan Content

Proteoglycan heterogeneity was demonstrated indirectly in rat mast cells by Enerbäck (1966b), who showed that CTMC, but not MMC, could be stained with safranin in the alcian blue/safranin staining sequence. Rat MMC, unlike rat dermal mast cells (Homer 1971, Enerbäck 1987, Enerbäck et al 1989) contain little or no heparin (Tas and Berndsen 1977), but instead were stated to contain predominantly chondroitin sulphate di-B (Stevens et al 1986b, Barrett and Pearce 1993, Schwartz 1994). However, at variance to this, Enerbäck et al (1985) have demonstrated biochemically that rat gastrointestinal MMC appear to contain chondroitin sulphate E.

As in the rat, human cutaneous mast cells contain heparin (Metcalfe et al 1980), with MMC originally being stated to contain chondroitin sulphate E alone (Gilead et al 1987). It is now

believed that all human mast cells in skin, lung and bowel contain heparin (Craig et al 1993), which supports previous findings suggesting that human lung mast cells contained both proteoglycans (Stevens et al 1988).

The CTMC subset in the mouse contains heparin (Schwartz 1994), whereas it is suspected that murine MMC do not contain heparin given that they cannot bind berberine sulphate dye (Kitamura et al 1987) and are alcian blue-positive but safranin-negative on sequential staining (Crowle and Phillips 1983).

Heparin is therefore the predominant proteoglycan of the CTMC subset in various species (Katz, Stevens and Austen 1985, Schwartz 1994), although it is not known whether this generalisation can be extended to include the ovine dermal mast cell population.

1.6.3. Arachidonic Acid Metabolism Product Heterogeneity

Mast cells from different sources also differ in their metabolism of arachidonic acid following activation, leading to the differential synthesis of prostaglandins [PG] (via the cyclooxygenase pathway) or leukotrienes [LT] (via the lipoxygenase pathway).

Cutaneous mast cells in man synthesize PGD_2 in preference to LTC_4 (Lawrence et al 1987, Benyon, Robinson and Church 1989; preparations $\leq$ 85% purity), which is similar to rat serosal mast cells following ionophore (Roberts et al 1979) or anti-IgE-mediated activation (Lewis et al 1982). Human uterine and lung mast cells produce equivalent quantities of PGD_2 and LTC_4 upon activation (Fox et al 1985, Massey et al 1991). However, studies on human dispersed (Leung et al 1987) or bronchoalveolar lavage-derived lung mast cells (Cohan et al 1989) suggest that PGD_2 synthesis predominates.

In vitro, rat BMMC and partially purified intestinal mast cells synthesize comparable amounts of PGD_2 , LTB_4 and LTC_4 upon IgE-dependent activation (Broide, Metcalfe and Wasserman 1988). In contrast, murine BMMC synthesize LTB_4 and LTC_4 in preference to PGD_2 (Razin

et al 1982, 1983), but this can be modulated by cytokines (particularly stem cell factor [SCF]) resulting in a more "CTMC"-like phenotype of PGD_2 generation (Murakami, Austen and Arm 1995).

Ovine CM-BMMC were shown to generate LTC_4 upon activation (Huntley et al 1992), although it is not known if ovine dermal mast cells would respond similarly.

1.6.4. Functional Heterogeneity

The differential response of mast cell populations *in vivo* or *in vitro* to chemical secretagogues and to the effect of anti-allergic drugs on mediator release has also been used to distinguish mast cell subsets in various species (reviewed in Cohan et al 1989, Foreman 1993, Barrett and Pearce 1993). The functional response of mast cells is the most clinically relevant of the methods used to define mast cell heterogeneity. In the series of studies described in this thesis three known mast cell secretagogues (compound 48/80 [48/80], substance P [sP] and calcium ionophore A23187 [A23187]) and one mast cell chymase (SMCP) were used in an attempt to activate ovine mast cells *in vivo* and *in vitro*.

Functional heterogeneity was originally demonstrated in the rat by injecting the polycationic secretagogue 48/80, which depleted dermal but not gastrointestinal mast cells [the MMC phenotype] (Enerbäck 1966c). Compound 48/80 is a mixture of polymers which evoke histamine release from mast cells in a non-cytotoxic process (Johnson and Moran 1969) that requires the presence of extracellular calcium (Foreman and Mongar 1973). It is postulated that, as suggested for substance P, 48/80 may activate, via a peptidergic pathway, mast cell G_i-like proteins in a receptor-independent but membrane-assisted process that is limited to serosal mast cells (Mousli et al 1994). Compound 48/80 is generally an activator of the serosal or CTMC phenotype (Table 1.3(a)), although one exception is the cow, as dispersed cutaneous mast cells could not be activated by 48/80 (Hunt et al 1991, Table 1.3(b)).

The neuropeptide substance P (reviewed in Regoli, Bondon and Fanchére 1994) will activate mast cells, but only at relatively high biological concentrations (10^{-6}M, 10^{-5}M)(Regoli, Bondon and Fanchére 1994). Substance P will activate cutaneous mast cells in man, but does not activate cells of the MMC phenotype (Table 1.4). This rule holds for most species, although sP can activate the MMC of the rat intestine (Befus et al 1986; Table 1.4).

Calcium ionophore A23187 (A23187) is considered a universal activator of mast cell populations *in vivo* and *in vitro* (Table 1.5), although different populations may show a gradation of response (Pearce 1986). As A23187 acts by increasing intracellular calcium levels (Foreman, Mongar and Gomperts 1973), responses can be evoked in other cell types (Amon et al 1994) and therefore its action is not mast cell-specific. Thus, although useful as a mast cell activating agent, it is of limited use in helping to define mast cell heterogeneity. A23187 is, however, the only chemical secretagogue to have successfully activated ovine CM-BMMC (Huntley et al 1992).

Chymase can activate rat serosal mast cells *in vitro* in an active site-dependent process (Schick and Austen 1986), although the exact mechanism of this activation is unknown. Both RMCP-I and RMCP-II can evoke β-hexosaminidase release from rat CM-BMMC, although the extent of release was less than that from isolated rat peritoneal mast cells (Gardner 1990).

Thus, by comparing the effects of the four agents discussed above on ovine mast cell populations *in vivo* and *in vitro*, it was hoped to further elucidate ovine mast cell phenotypic heterogeneity.

Mast Cell Population	Human	Rat	Mouse	Hamster	Guinea Pig	Cow	Sheep
CTMC (Connective Tissue Mast Cell Phenotype)	Schwartz 1989	Enerbäck 1966c, Stevens & Austen 1989, Tainsh & Pearce 1992	Katz, Stevens & Austen 1985				
Skin	Benyon, Lowman & Church 1987, Kaminer et al 1991	Pearce 1986, Barrett & Pearce 1993					
Peritoneal		Burwen 1982, Befus et al 1986	Barrett & Pearce 1993		Pearce 1986 (variable response)		
Thymus/ fibroblast culture		Ishizaka et al 1977					
Peritoneal/ fibroblast coculture		LeviSchaffer and Riesel 1989					
IL-3 BMMC			Chiu & Burrall 1990				

Table 1.3(a). Studies which have reported or cited compound 48/80-induced mast cell activation in various mast cell populations from various species. (Thymus/fibroblast culture indicates mast cell culture derived from thymus cells grown with fibroblast monolayers. Peritoneal/fibroblast coculture indicates peritoneal mast cells grown in coculture with 3T3 fibroblasts. IL-3 BMMC indicates bone marrow cells grown in the presence of IL-3).

Mast Cell Population	Human	Rat	Mouse	Hamster	Guinea Pig	Cow	Sheep
MMC (Mucosal Mast Cell Phenotype)	Schwartz 1989	Enerbäck 1966c, Foreman 1993					
Lung	Lawrence et al 1987, Benyon, Lowman & Church 1987, Cohan et al 1989, Tainsh et al 1991					Hunt et al 1991	
Intestine	Lawrence et al 1987, Cohan et al 1989	Enerbäck 1966c, Befus et al 1986, Miller 1993a					
Skin						Hunt et al 1991	
All mast cells					Pearce 1986, Foreman 1993		
CM BMMC			Sredni et al 1983				
Umb.cord + 3T3 culture	Ishizaka, Furitsu & Inagaki 1991						

Table 1.3(b). Studies which have reported or cited a lack of compound 48/80-induced mast cell activation in various mast cell populations from various species. (CM-BMMC indicates bone marrow cells grown in the presence of conditioned medium. Umb.cord + 3T3 indicates mast cell culture derived from umbilical cord blood mononuclear cells grown with 3T3 fibroblasts).

Mast Cell Population	Human	Rat	Mouse	Hamster	Guinea Pig	Cow	Sheep
Positive	**Responses**						
CTMC (Connective Tissue Mast Cell Phenotype)		Tainsh & Pearce 1992					
Skin	Benyon, Lowman & Church 1987, Church et al 1989, Cohan et al 1989, Foreman 1993	Galli 1990	Yano et al 1989			Hunt et al 1991	
Peritoneal		Befus et al 1986, Foreman 1993					
Intestine		Befus et al 1986, Miller 1993a					
Umb.cord + 3T3 culture	Ishizaka, Furitsu & Inagaki 1991						
Negative	**Responses**						
Lung	Church et al 1989, Cohan et al 1989					Hunt et al 1991	
Intestine	Lawrence et al 1987						
All mast cells					Foreman 1993		
CM BMMC		Broide, Metcalfe & Wasserman 1988					

Table 1.4. Studies which have reported or cited the presence or absence of substance P-induced mast cell activation in various mast cell populations from various species. (Umb.cord + 3T3 indicates mast cell culture derived from umbilical cord blood mononuclear cells grown with 3T3 fibroblasts. CM-BMMC indicates bone marrow cells grown in the presence of conditioned medium).

Mast Cell Population	Human	Rat	Mouse	Hamster	Guinea Pig	Cow	Sheep
Serosal		Pearce 1986					
Skin	Benyon, Lowman & Church 1987, Tainsh et al 1991, Amon et a1 1994, Kaminer et al 1991	Pearce 1986*	Yano et al 1989			Hunt et al 1991	
Peritoneal		Befus et al 1986	Pearce 1986	Pearce 1986			
Mesentery		Pearce 1986*			Pearce 1986*		
Lung	Cohan et al 1989, Tainsh et al 1991	Pearce 1986*			Pearce 1986*	Hunt et al 1991	
Intestine	Cohan et al 1989	Befus et al 1986, Pearce 1986*					
CM BMMC culture		Broide, Metcalfe & Wasserman 1988	Sredni et al 1983				Huntley et al 1992
IL-3 BMMC culture			Chiu & Burrall 1990				
Umb.cord + 3T3 culture	Ishizaka, Furitsu & Inagaki 1991						

Table 1.5. Studies which have reported or cited calcium ionophore (A23187)-induced mast cell activation in various mast cell populations from various species. (CM-BMMC and IL-3 BMMC indicates bone marrow cells grown in the presence of conditioned medium and IL-3 respectively. Umb.cord + 3T3 indicates mast cell culture derived from umbilical cord blood mononuclear cells grown with 3T3 fibroblasts. Pearce 1986* indicates that the cell population was activated, but could be considered hyporesponsive relative to other cell types).

1.7. Mast Cell Development *In Vitro* And *In Vivo*

It is considered that mast cell development and heterogeneity *in vitro* and *in vivo* is closely regulated by a range of cytokines (reviewed in Galli 1990, Schwartz 1994).

1.7.1. *In Vitro*

1.7.1.1. Conditioned Medium And Interleukin-3

Early *in vitro*-derived mast cells in rats and mice were generated from thymic (Ginsburg 1963, Ishizaka et al 1976, 1977) or lymph node-derived (Ginsburg and Lagunoff 1967, Denburg, Befus and Bienenstock 1980) cell populations. Subsequently, mast cells were generated *in vitro* from bone marrow cells of the mouse (Nagao, Yokoro and Aaronson 1981, Razin, Cordon-Cardo and Good 1981, Sredni et al 1983) and rat (Haig et al 1982) using conditioned media derived from stimulated splenocytes and T cells respectively.

T cells were shown to synthesise a factor that evoked proliferation of cloned mast cells (Nabel et al 1981), and the factor in conditioned media from concanavalin A-stimulated lymphocytes that possessed mast cell growth activity was demonstrated to be interleukin-3 (IL-3) (Ihle et al 1983). When grown in the presence of IL-3, bone marrow cells from the mouse (Ghiara et al 1985, Rennick et al 1985, Chiu and Burrall 1990) and rat (Haig et al 1988a) differentiate into mast cells. However, in human bone marrow cultures supplemented with IL-3, the production of basophils is favoured (Kirshenbaum et al 1989, Valent et al 1989), although small numbers of mast cells can be generated (Kirshenbaum et al 1989, 1992). IL-3 is considered an "early acting" cytokine in haematopoiesis (Ogawa 1993), targeting haematopoietic stem cells or multipotential progenitors (Arai et al 1990). Yet, uniquely in the case of the mast cell, IL-3 is believed to support lineage-restricted development (Ogawa 1993). Human mast cells are, however, unresponsive to IL-3 (Agis and Valent 1995), IL-3 binding sites on these cells being as yet undetected (Valent et al 1990).

In the sheep, as in the mouse and rat, mast cells can be produced from bone marrow grown in the presence of lymphocyte-derived conditioned media from nematode-infected sheep (Haig et al 1988b, Huntley et al 1992). These CM-BMMC developed rapidly after two weeks of culture and could be grown for two to three months *in vitro*, mast cells accounting for 90% of cultured cells by day 28 (Haig et al 1988b). The CM-BMMC were ultrastructurally immature, and contained arylsulfatase, β-hexosaminidase, dopamine and SMCP (Huntley et al 1992). The cells could be activated by A23187 to induce release of arylsulfatase, β-hexosaminidase and SMCP and to generate LTC_4 (Huntley et al 1992). A second putative chymase (in addition to SMCP) was detected by Western blotting. The second immunoreactive band was not detected in blots from purified gastric MMC and it was suggested that CM-BMMC represented a mixed population of CTMC and MMC phenotypes (Huntley et al 1992).

IL-3 promotes the proliferation of precursor erythroid cells, megakaryocytes, macrophages, neutrophils, eosinophils and mast cells. The IL-3 receptor expresses both high and low affinities for IL-3 (i.e. dual affinity; Foxwell, Barrett and Feldmann 1992). The α-chain of the heterodimeric receptor expresses low affinity binding, high affinity binding occurring as a result of the association of each α-chain with a larger β-chain (Foxwell, Barrett and Feldmann 1992). The β-chain, which by itself cannot bind IL-3, is also shared by the receptors for IL-5 and GM-CSF (Foxwell, Barrett and Feldmann 1992), all three receptors being members of the haematopoietic growth receptor family. IL-3, IL-5 and GM-CSF induce tyrosine phosphorylation on a similar set of cytoplasmic proteins (discussed in Foxwell, Barrett and Feldmann 1992), and this may be the reason that these cytokines are able to demonstrate apparently overlapping effects on different cellular compartments (reviewed in Lopez et al 1992). Transformed murine mast cell lines and purified peritoneal mast cells (Plaut et al

1989) produced IL-3 when activated by cross-linkage of FcεRI or by calcium ionophores. Murine CM-BMMC (Wodnar-Filipowicz, Heusser and Moroni 1989) and murine IL-3-dependent or -independent cloned mouse mast cells (Burd et al 1989) expressed increased levels of mRNA for IL-3 and also IL-3 bioactivity when stimulated through FcεRI. Recently, IL-3 and IFN-γ have been shown to modulate the release of serotonin and arachidonate (eicosanoids) from mouse peritoneal mast cells, IL-3 enhancing, while IFN-γ inhibits, their antigen-induced release (Holliday et al 1994). Thus, locally produced IL-3 could also play a role in the modulation of inflammatory mediators at sites of cutaneous inflammation and, importantly, the *in vivo* release of mast cell-derived IL-3 may facilitate the local proliferation of mast cells and help determine their final phenotype.

Latterly, the ovine IL-3 gene was cloned and the recombinant protein expressed (M^{c}Innes, Haig and Logan 1993). The exons of the ovine IL-3 gene share approximately 50% identity with those of man (M^{c}Innes 1993, M^{c}Innes, Haig and Logan 1993). Ovine IL-3 protein is 35% and 24% homologous in terms of predicted amino acid sequences with human and murine IL-3 respectively (M^{c}Innes 1993, M^{c}Innes, Haig and Logan 1993). Preliminary studies have suggested that rOv.IL-3 supports the development of cell colonies of mixed phenotype *in vitro* (including neutrophils, macrophages and eosinophils; Haig 1993). Additionally, this study suggests that rOv.IL-3 supports the development of mast cells from ovine bone marrow *in vitro* (Haig 1993). However, the kinetics of development of these cells, their mediator content, functional activity and likely phenotype were not determined.

1.7.1.2. Stromal Cell-Derived Factors (Stem Cell Factor And Nerve Growth Factor)

Stem cell factor (SCF), which was initially identified as a novel mast cell growth factor (Copeland et al 1990, Williams et al 1990, Zsebo et al 1990a) and ligand for the *c-kit* tyrosine kinase receptor (Huang et al 1990, Nocka et al 1990), has been shown to regulate mast cell

proliferation and maturation (Galli, Tsai and Wershil 1993), and specifically to act synergistically with IL-3 to promote the growth of both rat (Haig et al 1994) and mouse (Tsai et al 1991a) IL-3-derived BMMC. In the rat, the addition of IL-3 or CM to peritoneal mast cells (CTMC) grown in the presence of SCF *in vitro* resulted in a subpopulation of CTMC expressing and storing RMCP-II (Haig et al 1994). However, in the same study, SCF favoured the maintenance of RMCP-I in CTMC and rat BMMC grown in the presence of SCF demonstrated a reduction in RMCP-II levels without a concomitant increase in RMCP-I (Haig et al 1994). Thus, the presence of SCF can influence the production of proteinases in these two cell types. SCF alone can stimulate small numbers of murine BMMC (Boswell et al 1990, Migliaccio et al 1991). SCF was also shown to induce the development of human mast cells from both bone marrow and peripheral blood mononuclear cells (Valent et al 1992) and from umbilical cord blood cells (Mitsui et al 1993), although other studies suggest it cannot, in the absence of other factors, induce mast cell growth (Grabbe et al 1994). The ovine stem cell factor gene has recently been cloned (C.M^{c}Innes, *personal communication*) and its subsequent expression should provide recombinant ovine stem cell factor which will allow investigation of its role in ovine mast cell development *in vivo* and *in vitro*. In addition to the interplay between interleukins and SCF, nerve growth factor (NGF) can also induce murine CTMC-type mast cell growth from bone marrow cells (Matsuda et al 1991).

1.7.1.3. Other Cytokines

Interleukin-4, although having no direct effect on mast cell growth, synergizes with IL-3 to promote growth of BMMC in the mouse (Smith and Rennick 1986) and, similarly, IL-10 in combination with either IL-3 or IL-4 has been shown to support the growth of murine mast cells *in vitro* (Thompson-Snipes et al 1991). Interleukin-9, produced by activated T_{H2} clones *in vitro* (Renauld et al 1995), can enhance the proliferation of murine mast cell lines induced by IL-3 or IL-4 (Hültner et al 1989, Moeller et al 1989, Hültner et al 1990), the latter

cytokines being able to inhibit the IL-9-induced expression of MMCP-1, MMCP-2 and MMCP-4 (Eklund et al 1993). Recently, several cytokines (IL-3, IL-4 and IL-10) have been demonstrated to be necessary for stem cell factor-dependent growth of murine mast cell progenitors (Rennick et al 1995). However, IL-4, IL-9 and IL-10 fail to support the growth of human mast cells *in vitro* (Agis and Valent 1995).

1.7.1.4. Use Of Fibroblast Co-Culture Systems

Co-culture systems, where progenitor cells are cultured in the presence of fibroblasts, have been used extensively to generate and investigate mast cells from rodents (Ginsburg, Ben-Shahar and Ben-David 1982, Levi-Schaffer et al 1985 and 1986, Nakamura et al 1994) and man (Furitsu et al 1989, Ishizaka, Furitsu and Inagaki 1991, Irani et al 1992) *in vitro*. Specifically, such co-culture experiments have been used to provide evidence that the local tissue microenvironment may regulate the phenotype of mast cells in tissues (Levi-Schaffer 1985, 1986; Dayton et al 1988). For example, the study by Dayton et al (1988) indicated that murine IL-3-dependent BMMC could alter their proteoglycan content and therefore acquire characteristics of the connective tissue mast cell subset in the presence of fibroblasts. This study assumed that the IL-3-dependent BMMC population was analogous to the MMC population. However, it is known that the murine IL-3-dependent BMMC population is heterogeneous (discussed later; Newlands et al 1991) and, therefore, these results may merely represent the subsequent selection by fibroblast co-culture of a subset of the original BMMC. Thus, the interpretation of *in vitro* findings based on heterogeneous cell populations whose similarities to *in vivo* cell populations are open to speculation must be viewed critically.

It has been demonstrated that the survival of rat BMMC when co-cultured with murine 3T3 fibroblasts is dependent upon fibroblast-derived stem cell factor (MacDonald 1994). Preliminary studies indicated that a well-granulated, adherent mast cell population developed

when ovine CM-BMMC were grown in a co-culture system (J.Huntley, *personal communication*). However, it is not known if the ovine CM-BMMC phenotype would be altered, as is suggested to occur with murine IL-3 BMMC (Levi-Schaffer et al 1986).

1.7.2. *In Vivo*

1.7.2.1. Stem Cell Factor

The importance of SCF in the development of CTMC and MMC populations *in vivo* was indicated by the lack of mast cells in WBB6F$_1$-*W/W^v* [*W/W^v*](Kitamura, Go and Hatanaka 1978) or WCB6F$_1$-*Sl/Sl^d* [*Sl/Sl^d*](Kitamura and Go 1979) mice, which lack *c-kit* receptor and SCF production respectively (reviewed in Nakano et al 1985, Nakano et al 1987, Galli, Tsai and Wershil 1993). SCF is produced *in vivo* by fibroblasts (Flanagan and Leder 1990, Nocka et al 1990), keratinocytes (Longley et al 1992), dermal endothelium (Weiss et al 1995), bone marrow stromal cells (Williams et al 1990), hepatocytes, thymic stromal cells, mast cells and Langerhans cells (Grabbe et al 1994) and is expressed either as a soluble or membrane-bound form (Anderson et al 1990, Flanagan, Chan and Leder 1991). The *c-kit* tyrosine kinase receptor is expressed on primordial stem cells, intrathymic stem cells, CD34$^+$ bone marrow cells, megakaryocyte precursors, erythroid precursors, granulocyte precursors, melanocytes and mast cells (Grabbe et al 1994). SCF at high concentrations can evoke histamine release from human skin mast cells, but not from human lung mast cells (Bischoff and Dahinden 1992, Columbo et al 1992). Daily subcutaneous injection of recombinant rat SCF (rrSCF) induces cutaneous mast cell development in the *Sl/Sl^d* mast cell-deficient mouse (Zsebo et al 1990b). Additionally, local administration of rrSCF in normal mice results in cutaneous CTMC development (Tsai et al 1991b), and intravenous rrSCF induces the development of both MMC and CTMC populations (Tsai et al 1991b, Newlands et al 1995). Systemic injection of anti-SCF antibody ablates the MMC population in normal rats but only partially depletes peritoneal cells and has only very limited effects on CTMC in other sites (Newlands

et al 1995). Similarly, in primates, mast cell numbers at various tissue sites increase following subcutaneous injection with recombinant human SCF, these falling again upon cessation of SCF treatment (Galli et al 1993). Mast cell hyperplasia in mice due to a primary *Trichinella spiralis* infection was abrogated by treatment with a monoclonal antibody to *c-kit* (Grencis et al 1993). Therefore, SCF and *c-kit* are closely associated with mast cell development *in vivo* and, from the evidence discussed above, also *in vitro*.

1.7.2.2. Cytokines

The subcutaneous injection of IL-3 in Rhesus monkeys results in a perivascular dermal cellular infiltrate (composed mainly of lymphocytes, monocytes and eosinophils) with increased numbers of mast cells (Volc-Platzer et al 1991), although the phenotype of these cells was not determined. This is of particular interest given that, as discussed above, only small numbers of mast cells can be generated by supplementing human bone marrow cultures with IL-3 (Kirshenbaum et al 1989, 1992). Given that human mast cells can be generated from peripheral blood mononuclear cells (Valent et al 1992) grown in the presence of SCF, the implication from these observations would be that a combination of growth factors are necessary to evoke human mast cell development. In the study by Volc-Platzer et al (1991) these may have been provided by cells in the local microenvironment activated by IL-3. This theory would be supported by the current lack of evidence for the presence of the IL-3 receptor on human mast cells (Valent et al 1990). Systemic administration of IL-3 evokes the generation of mast cells of an apparent MMC phenotype based on alcian blue staining in mice (along with cells from other haematopoietic lineages; Metcalfe et al 1986). IL-3 also acts as a signal for the expansion of the intestinal MMC population during parasitic infection *in vivo* (Abe and Nawa 1988).

Interestingly, following the observation that increased numbers of mast cells are associated with the inoculation of a keratinocyte-derived squamous cell carcinoma cell line (KCMH-1) in mice *in vivo*, a KCMH-1-derived factor has been identified that induces mast cell growth in conjunction with 3T3 fibroblasts *in vitro* that is not IL-3, IL-4, IL-9, IL-10 or SCF (Nakamura et al 1994, Yamamoto et al 1995).

The evidence discussed above suggests that a number of growth factors can favour the development of mast cells *in vitro* and *in vivo*. If this is coupled with the consideration of mast cell heterogeneity, then the crucial question is:- What actually regulates the development of tissue-specific mast cell phenotypes? For example, differences in the content of mast cell proteinases in different tissues (e.g. skin versus gut) may reflect differences in the role of the mast cell at these respective sites. Currently, there are two hypotheses to explain the regulation of tissue-specific mast cell phenotypes. The first hypothesis speculates that progenitors will only develop along a lineage-specific pathway once lodged into the appropriate tissue (i.e. CTMC progenitors would only develop in a CTMC site e.g. skin). The second hypothesis, for which there is supportive *in vivo* evidence, suggests that the mast cell precursor has the potential to express a variety of gene products and that this expression is regulated by microenvironmental factors in the vicinity of the precursor cell. Certainly, peritoneal mast cells from congenic normal $WBB6F_1$ (+/+) mice transplanted into the gastric mucosa of W/W^v mast cell-deficient mice, result in the development of MMC-like cells (Kitamura et al 1987). *In vitro* studies, where BMMC are grown in combinations of cytokines and the cells monitored for changes in proteinase phenotype, are faced with the problem of the initial cell population possibly being heterogeneous (as demonstrated in murine BMMC; Newlands et al 1991). Thus, apparently cytokine-induced alterations in proteinase expression could merely reflect the cytokine-dependent selection of different precursor cell subsets from the original heterogeneous population. For example, the MMCP-1 mRNA

transcript was stated to be undetectable in murine BMMC (it being stated to be a "late-expressed" proteinase), whereas the addition of IL-10 resulted in the expression of MMCP-1 mRNA (Ghildyal et al 1992). However, it is known that MMCP-1 can be detected by immunofluorescence and Western blotting and that the content of MMCP-1 of murine BMMC varies with culture conditions *in vitro* (i.e. IL-3 versus CM; Newlands et al 1991). Thus, the former study (Ghildyal et al 1992) may have merely selected a receptive subset of the heterogeneous murine BMMC population.

As stated previously, the relevance of *in vitro* studies based upon non-homogeneous cell populations must be viewed with scepticism based on the likely complex nature of phenotypic regulation *in vivo*. One substantial piece of evidence against mast cell phenotypic heterogeneity being derived from multiple progenitor populations is the recent study by Gurish et al (1995), which demonstrated the acquisition and loss of specific proteinases in an immature *v-abl* transformed mast cell line (V3-MC) injected intravenously into BALB/c mice following infiltration of these cells into liver, spleen and intestine. Thus, cultured V3-MC (based on immunohistochemical and RNA blot data) demonstrated MMCP-5 and MMCP-6, whereas liver infiltrates demonstrated MMCP-1 to MMCP-7 and intestinal infiltrates MMCP-1 and MMCP-2 two to three weeks after administration (Gurish et al 1995). Thus, although further *in vivo* investigations need to be undertaken, the scant evidence available suggests against a multiple progenitor cell population and favours the differentiation and maturation of progenitor cells being regulated primarily by the tissue microenvironment.

1.8. The Potential Function Of Cutaneous Mast Cells: Evidence To Support A Role In Cutaneous Inflammation

1.8.1. Cell Adhesion Molecules And Cell Trafficking

In inflammatory disorders of the skin many of the histopathological changes are due to increased immune cell trafficking, this invariably appearing initially as a perivascular

accumulation of leukocytes (Walsh, Lavker and Murphy 1990). Changes in cell trafficking can result from mast cell-derived mediators activating endothelial cells and other cell types, and a brief discussion of adhesion molecules on cells and endothelium (reviewed in Nickoloff 1990, Springer 1994) is necessary before discussing the respective actions of the mediators themselves.

Endothelial-leukocyte adhesion molecule-1 (ELAM-1) is a lectin-like cell surface glycoprotein that mediates adhesion of blood leukocytes to the endothelial lumenal membrane, ELAM-1 being expressed exclusively by cytokine-activated endothelial cells (Bevilacqua et al 1987). Expression of ELAM-1 in cultured endothelial cells is induced by IL-1 and TNF-α, and is maximal at 6 hours and receding by 24 hours (Pober et al 1986). In human skin, ELAM-1 expression is confined to post capillary venules (the skin equivalent of high endothelial venules)(Klein et al 1989). ELAM-1 binds strongly to a carbohydrate ligand on neutrophils (reviewed in Springer 1994) and, to a lesser degree, to monocytes and lymphocytes (Bevilacqua et al 1987). VCAM-1 (vascular cell adhesion molecule-1) is also expressed on cytokine-activated (e.g. by TNF-α; MacKay and Imhof 1993) endothelial cells (Osborn et al 1989), but binds selectively to lymphocytes and not to neutrophils (Osborn et al 1989). Intercellular adhesion molecule-1 (ICAM-1) is expressed by endothelial cells, lymphocytes, monocytes and fibroblasts *in vivo* (Dustin et al 1986); high levels of ICAM-1 expression are induced by cytokines (e.g. IL-1), are maximal at 24 hours, and are maintained for 72 hours *in vitro* (Dustin et al 1986, Wawryk et al 1989). An integrin, leukocyte function-associated antigen (LFA-1), expressed on all blood mononuclear cells, acts as a ligand for ICAM-1 (Preito et al 1988). Leukocyte adhesion to endothelium occurs in three stages (Fig.1.1, reviewed in Springer 1994). The initial attachment of leukocytes to a vessel wall is mediated by selectins (P- or E-selectin [ELAM-1] on endothelium, L-selectin on leukocytes), leading to "rolling" adhesion. Activation of leukocyte integrins results in firm attachment of the

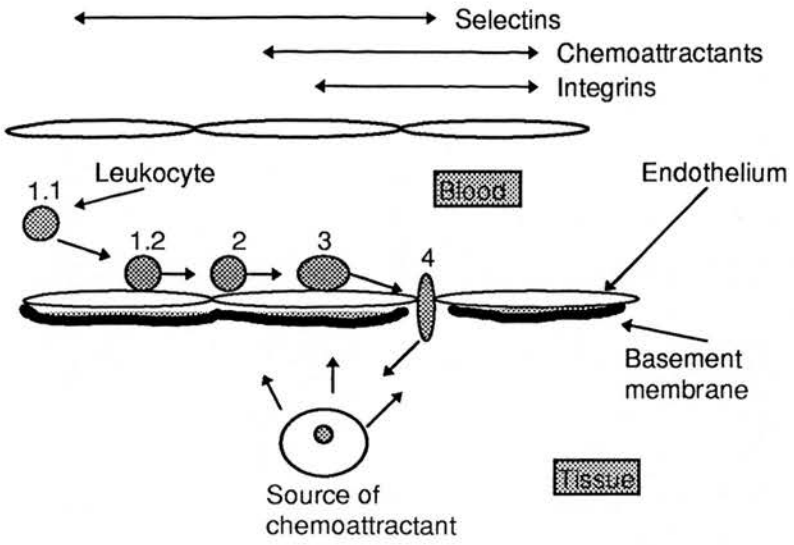


Figure 1.1. Diagram of the three stages involved in cell trafficking. **Stage 1**: The initial attachment of leukocytes (1.1) to a vessel wall is mediated by selectins (P- or E-selectin on endothelium, L-selectin on leukocytes), leading to "rolling adhesion" (1.2). **Stage 2**: The activation of leukocyte integrins (2 and 3) results in firm attachment of the leukocyte to endothelium via LFA-1 and ICAM-1 or VCAM-1 and VLA-4 (integrin). **Stage 3**: The leukocyte then alters shape and migrates through the endothelium, in response to chemoattractants.

leukocyte to endothelium via LFA-1 and ICAM-1 or VCAM-1 and VLA-4 (integrin). The leukocyte then alters shape and migrates through the endothelium, due to chemoattractants (Colditz 1991a, Springer 1994).

Mast cell-derived mediators, including histamine, prostaglandins and leukotrienes, proteoglycans, proteinases and cytokines may all contribute to the vascular and cellular responses that accompany cutaneous inflammation. Each mediator, and its prospective role in cutaneous inflammation, will be briefly discussed below.

1.8.2. Mast Cell-Derived Mediators

1.8.2.1. Histamine

Histamine (β-imidazolyethylamine) is stored, preformed, in mast cell granules, bound to the anionic side chains of proteoglycans from which it is released upon secretion (Falus and Merétey 1992). Once released it diffuses rapidly into surrounding tissues, appearing in blood within $2^1/_2$ minutes and returning to baseline levels within 15 to 30 minutes (White, Slater and Kaliner 1987). Histamine evokes increased vascular permeability (following endothelial cell contraction and junctional widening) by causing leakage from small and medium sized venules, these vessels having a greater concentration of histamine receptors than either arteriolar or capillary endothelium (Pasyk and Cherry 1990).

In skin, all of the cardinal features of urticaria (vasodilation, increased vascular permeability and pruritus) can be mediated via the H_1 receptor (White 1990). However the classical "triple response" effects of histamine in skin (i. Central erythema due to histamine-induced vasodilation, ii. Peripheral erythema due to neuropeptide-mediated axon reflex vasodilation, iii. Weal formation due to increased vasopermeability by histamine) require a combination of both H_1- and H_2-receptor antagonists to be completely abrogated (Robertson and Greaves 1978).

In addition to the effects on vasopermeability in skin, histamine can also regulate the production of cytokines (e.g. i. Down-regulation of IL-1 and TNF-α production from human monocytes, ii. Potentiation by IL-1 of the effect of histamine-induced release of prostaglandins by endothelial cells (Revtyak et al 1988), iii. Inhibition of T cell proliferation and immunoglobulin production, iv. Enhancement (via the H_1 receptor) or inhibition (via the H_2 receptor) of the generation of complement and acute phase proteins (reviewed in Falus and Merétey 1992)). Histamine acts upon endothelial cells via H_2 receptors to release PGI_2 (prostacyclin), which is a potent inhibitor of platelet aggregation (Harvima and Schwartz 1993).

Plasma leakage in response to intradermal injection of histamine in ovine skin occurs maximally by 10 minutes following injection, and effectively ceases 30 minutes thereafter (Colditz 1991b), although no histological analysis of the intradermal test site for the presence of a cellular infiltrate was undertaken. As mast cells are in close proximity to dermal blood vessels, a rapid increase in vasopermeability can occur due to the release of histamine and other newly-generated lipid mediators upon activation (see below), and other broader effects on the skin immune system can be evoked.

1.8.2.2. Prostaglandins And Leukotrienes

As discussed above, activated mast cells can generate PGD_2, LTB_4, LTC_4 and LTD_4 from arachidonic acid. PGD_2 (produced predominantly by the CTMC subset of most species) is a potent vasodilator, whereas LTC_4 and LTD_4 evoke a short-lived vasoconstriction (Dahlén et al 1981) followed by an increase in vascular permeability (Orange and Austen 1972, Dahlén et al 1981). In the skin, these mediators may act synergistically (Soter et al 1983), thereby augmenting the LTB_4-mediated accumulation of neutrophils. PGD_2 levels are increased in blood following urticarial reactions (Heavey et al 1986). The weal response to LTC_4 and

LTD_4 is of greater duration (weal 2 hours, erythema 6 to 8 hours; Soter et al 1983) than that due to histamine or PGD_2, the mechanism for this being due to arteriolar constriction and then vasodilation, venular permeability being subsequently increased (discussed in Harvima and Schwartz 1993).

In sheep, as in man (Soter et al 1983), intradermal injection of LTB_4 evokes dermal neutrophil influx (Colditz and Movat 1984, Colditz and Watson 1992). LTB_4 failed to evoke ovine neutrophil migration *in vitro* (Mulder and Colditz 1993), the *in vivo* LTB_4-induced neutrophil influx depending neither on the induction of adhesion molecules nor on synthesis of IL-8 (Mulder and Colditz 1993). One possible mechanism for the observed neutrophil influx could be direct activation of neutrophils by LTB_4 (Krogsgaard Thomsen 1991), resulting in the development of neutrophil-induced oedema (Wedmore and Williams 1991) and subsequent increased neutrophil influx. LTB_4 also potentiates the differentiation of IL-4-responsive B lymphocytes, which is of particular relevance as activated mast cells are one of the major sources of IL-4 in skin (Bradding et al 1992, Horsmanheimo et al 1994), this possibly contributing to the regulation of immunoglobulin production (particularly IgE).

Although it is not known which of the arachidonic acid metabolites can be produced by activated ovine dermal mast cells, it is known that ovine CM-BMMC can be activated by A23187 to release LTC_4 (Huntley et al 1992). This indicates that mast cell-derived arachidonic acid products are likely to contribute to vasopermeabiltiy changes in ovine cutaneous inflammation.

1.8.2.3. Proteoglycans

As discussed previously, heparin is the predominant proteoglycan present in CTMCs. Both tryptase (Caughey et al 1988a) and chymase (Sayama et al 1987) are bound to heparin upon release, this binding enhancing the activity of human chymase (M^{c}Euen, Sharma and Walls

1995) and stabilising tryptase (Schwartz and Bradford 1986). Heparin may also modulate the cell adhesion properties of vitronectin, fibronectin and laminin (Kjellen and Lindahl 1991), activated mast cells being capable of adherence to both vitronectin (Bianchine, Burd and Metcalfe 1992) and fibronectin (Dastych et al 1991). Heparin also possesses anti-coagulant and anti-complement properties. Therefore, dermal mast cell-derived heparin release would be pro-inflammatory overall, although it may also have a role in cell repair, as capillary endothelial cells can be activated by heparin *in vitro* (Harvima and Schwartz 1993).

1.8.2.4. Mast Cell Proteinases

1.8.2.4.1. Biological substrates

Binding of human chymase to heparan sulphate (a component of the basement membrane) and to the basement membrane in skin itself was demonstrated immunohistochemically (Sayama et al 1987), this process leading to degradation of the epidermal-dermal junction (Briggaman et al 1984) and subsequent dermatopathology (Schechter 1989) that is usually associated with immune-mediated disease, particularly bullous pemphigoid (Goldstein, Wasserman and Wintroub 1989). Chymases can also degrade the interstitial matrix in skin (Sage, Woodbury and Bornstein 1979, Seppa, Vaananen and Korhonen 1979), which may imply a role for chymases in cutaneous inflammation and repair.

Human tryptase has a relatively restricted protein substrate specificity, leaving most uncleaved (Schwartz 1995). For example, tryptase is unable to directly activate procollagenase nor degrade proteoglycan or collagen (Johnson and Cawston 1985), although it converts prostromelysin to stromelysin (matrix metalloproteinase 3) which in turn activates latent collagenase (Gruber et al 1989). Given that tryptase can also activate fibroblasts (Ruoss, Hartmann and Caughey 1991), tryptase may be involved in the repair and resolution of cutaneous inflammatory responses. Chymase, released following mast cell activation, has also been shown to be responsible for alterations in the coherent structure of fibroblast

monolayers *in vitro* (Ginsburg et al 1989). A putative role for chymase in enhancing the spread of transudate during inflammatory responses was speculated (Ginsburg et al 1989). Additionally, activated mast cells have recently been shown to be fibrogenic for 3T3 fibroblasts (Levi-Schaffer and Rubinchik 1995). This is supported by the observation that epithelial permeability can be markedly increased in an *ex vivo* rat intestinal perfusion system by RMCP-II (Scudamore et al 1995). Chymases may be able to up-regulate mast cell responses, as rat chymase can activate mast cells *in vitro* (Schick, Austen and Schwartz 1984, Schick and Austen 1986). Tryptase may also degrade neuropeptides (discussed later), generate C3a (Schwartz et al 1983) and possess anticoagulant attributes due to fibrinogen degradation (Schwartz et al 1985).

Enzymatic degradation is considered one of the major methods for peptide inactivation and thus for the regulation of neuropeptide-induced inflammatory responses *in vivo* (Tam 1995). Substance P can be degraded by dog (Caughey et al 1988d), rat (Le Trong, Neurath and Woodbury 1987), human (Urata et al 1990) and ovine (SMCP; Pemberton, Huntley and Miller, *submitted*) chymases, although tryptase has no effect on sP. Chymases can degrade the vasodilatory peptides bradykinin and kallidin (Reilly, Schechter and Travis 1985) and convert angiotensin I to angiotensin II (a vasoconstrictor) dependent on the source of chymase (Reilly et al 1982, Wintroub et al 1984, Urata et al 1990). Vasoactive intestinal peptide (VIP) is also degraded by dog chymase and tryptase (Caughey et al 1988d). Tryptase can also degrade CGRP, but has no effect on neurokinins A or B (Tam and Caughey 1990, Harvima and Schwartz 1993). It is also known that the activity of CGRP and VIP declines upon hydrolysis by tryptase (Walls et al 1992, Franconi et al 1989). The possible contribution of the mast cell (and particularly mast cell proteinases) to cutaneous neurogenic inflammation due to the close anatomical relationship between mast cells and cutaneous nerves (Wiesner-Menzel et al 1981) has been widely reviewed (Goetzl et al 1986, Baraniuk, Kowalski and

Kaliner 1990, Foreman 1993, Fantini et al 1995). However, no study has definitively described the cleavage of a peptide by a mast cell proteinase following mast cell activation *in vivo*. Intradermal injection of sP attenuated the prolonged vasodilation induced by injection of CGRP in human skin and this was postulated to be due to the release of proteases from sP-activated mast cells (Brain and Williams 1988), although this was not conclusively proven. It is known from studies in mast cell-deficient WBB6F$_1$-*W/W^v* and WCB6F$_1$-*Sl/Sl^d* mice that intradermal injection of sP evokes both an increase in vascular permeability and granulocyte influx that is mast cell dependent (Matsuda et al 1989, Yano et al 1989). (The granulocyte infiltration is, in part, due to direct activation of vascular endothelial cells by the C-terminal end of the sP peptide [Iwamoto et al 1992, Tomoe et al 1992] and expression of ICAM-1 [Nakagawa, Sano and Iwamoto 1995]). However, it is not known what contribution is played by mast cell proteinases in this sP-induced response. Further evidence which implies, but does not define, a role for mast cell proteinases in neurogenic inflammation is suggested by immunohistological studies of dermatoses purported to have a neurogenic component. For example, the number of tryptase-containing mast cells are increased in lesions of psoriasis (Harvima et al 1993), the tryptase being in an active form at all levels within the skin (Harvima et al 1989). Contacts of neuropeptide-containing sensory nerves with tryptase-containing mast cells are also increased in psoriasis (Harvima et al 1994), the nerve fibres staining positively for sP and CGRP, but not for VIP, in lesional skin (Naukkarinen et al 1994). Tryptase also apparently evokes histamine release from ovine dermal mast cells *in vivo* (Molinari et al, *in press*). There is thus some evidence for a role for mast cells and their granule proteinases to contribute to neurogenic inflammation, although the extent of this involvement is still open to debate (Kowalski and Kaliner 1988) and direct evidence *in vivo* is lacking.

1.8.2.4.2. Cutaneous responses

When injected intradermally, human chymase and tryptase preparations increase vascular permeability and leukocyte infiltration in rabbit skin (Fräki 1977). When human lung tryptase was injected intraperitoneally in mice, there was an influx of neutrophils (Walls et al 1994). Similarly, dermal neutrophil accumulation was induced apparently equally by intradermal injection of human chymase or tryptase in guinea pig skin (Walls et al 1994), indicating that mast cell chymase and tryptase release in skin may be associated with neutrophil recruitment. Rat chymase was also shown to cause pruritus when injected into human subjects (Hägermark, Rajka and Bergqvist 1972) and the intradermal injection of canine chymase potentiated histamine induced weal formation in the dog (Rubenstein et al 1990). Recently, in ovine skin, intradermal injection of tryptase isolated from a human neoplastic mast cell line (HMC-1) was associated with a histamine-mediated weal response that was increased in magnitude by the co-injection of heparin (Molinari et al, *in press*), which indicates that (as discussed previously), activity of mast cell proteinases may be modulated by the presence of heparin (M^{c}Euen, Sharma and Walls 1995).

1.8.2.4.3. Effects on cytokines

Human chymase converts pro-IL-1β to active IL-1 (Kupper et al 1990, Mizutani et al 1991) which, amongst many actions (see below) can directly activate vascular endothelial cells (Pober 1988). Human chymase has been shown to inactivate IL-4 (Delara et al 1994a, Delara et al 1994b).

The evidence provided above suggests that mast cell tryptase and chymase may contribute to cutaneous inflammatory responses. The function of these proteinases may involve vascular and epithelial permeability changes as suggested by the work, *in vivo*, of Rubenstein et al (1990) and Scudamore et al (1995). Further suggestive evidence of a pro-inflammatory role in cutaneous inflammation arises from the ability of chymase to generate IL-1 (Kupper et al

1990, Mizutani et al 1991), and development of dermal neutrophilic infiltrates following injection of proteinases *in vivo* (Walls et al 1994).

Whether proteinases activate endothelium directly via proteinase-activated receptors (Altieri 1995, Coughlin 1994) to promote this infiltration is open to speculation, but this would be an additional mechanism by which proteinases could modulate inflammatory responses. The role of proteinases in neurogenic inflammation is currently open to debate, although there is supportive data associating mast cells with neurogenic responses. Currently, relatively few biological substrates for mast cell chymase and tryptase have been identified *in vivo*. However, those substrates identified so far suggest possible effects for these proteinases on the extracellular cell matrix, on the basement membrane and on fibroblasts, indicating a role in the modulation and resolution of inflammatory responses in skin.

1.8.2.5. Mast Cell-Derived Cytokines

Mast cells, following activation by immunological or non-immunological stimuli, can generate or release various cytokines (reviewed in Gordon, Burd and Galli 1990), which are now thought to play a major role in the pathogenesis of cutaneous inflammation (reviewed in Harvima et al 1994). Although various mast cell-derived cytokines may participate in cutaneous responses (Table 1.6), the two of most probable relevance to the studies described in this thesis are tumour necrosis factor-α (TNF-α) and interleukin-8 (IL-8), these being discussed in detail below. However, it should be remembered that many other cell types in skin can generate cytokines (e.g. keratinocytes; Luger and Schwarz 1990, M^{c}Kay and Leigh 1991) and thus the mast cell may be a relatively minor contributor to the overall production of cytokines in skin.

Cytokine	Evidence for mast cell-derived mRNA or bioactivity	Evidence for a role in cutaneous responses *in vivo*
Interleukin-1 (IL-1)	Burd et al 1989	Dowd, Camp and Greaves 1989 Cybulsky, Colditz and Movat 1986 Colditz and Watson 1992
IL-3	Burd et al 1989 Wodnar-Filipowicz, Heusser and Moroni 1989	Volc-Platzer et al 1991
IL-4	Plaut et al 1989	Kay et al 1991 Dvorak et al 1994
IL-5	Burd et al 1989 Plaut et al 1989	Kay et al 1991
IL-6	Burd et al 1989 Plaut et al 1989	Grossman et al 1989
Basic fibroblastic growth factor	Qu et al 1995	Qu et al 1995
Transforming growth factor-β	Gordon and Galli 1994	Roberts et al 1986 Gordon and Galli 1994
"C-C" chemokines	Burd et al 1989 Selvan, Butterfield and Krangel 1994	Alam et al 1994
GM-CSF	Burd et al 1989 Wodnar-Filipowicz, Heusser and Moroni 1989	Meade et al 1993

Table 1.6. Evidence supporting the mast cell origin of selected cytokines, and also for the participation of these cytokines in cutaneous responses *in vivo*.

1.8.2.5.1. TNF-α

TNF-α is a potent immunoregulatory cytokine produced by many cutaneous cells, including mast cells, keratinocytes and Langerhans cells (reviewed in Le and Vilcek 1987, Piguet 1993). Mast cells contain pre-formed stores of TNF-α that is available for immediate release upon activation (Plaut et al 1989), mast cells being the prominent TNF-α-containing cell in human dermis (Walsh, Lavker and Murphy 1990). The injection of TNF-α into human (Groves et al 1995) or ovine (Colditz and Watson 1992) skin is pro-inflammatory, evoking a marked dermal neutrophilic infiltrate. TNF-α failed to induce ovine neutrophil migration *in vitro* (Mulder and Colditz 1993). IgE-dependent activation of mast cells induces release of pre-formed stores of TNF-α and also induces both TNF-α mRNA production and TNF-α synthesis (Gordon and Galli 1990a). Human dermal mast cells activated by morphine sulphate, release preformed TNF-α with immunoreactivity for TNF-α declining within 45 minutes (Walsh et al 1991). In the same study, ELAM-1 expression was induced as a direct consequence of mast cell-derived TNF-α release (Walsh et al 1991), and in human skin ELAM-1, VCAM-1 and ICAM-1 expression was induced by TNF-α injection (Groves et al 1995). Using the W/W^v mast cell-deficient mouse, almost all of the neutrophil infiltration occurring in IgE-dependent passive cutaneous anaphylaxis (PCA) reactions was shown to be mast cell dependent (Wershil et al 1991). Using an anti-TNF-α antibody, mast cell-dependent leukocyte infiltration was reduced by about 47% in this study (Wershil et al 1991). Thus, mast cell-derived TNF-α plays a prominent role in the dermal cellular influx that occurs following mast cell activation. Furthermore, dexamethasone and cyclosporin A reduce mast cell-derived TNF-α mRNA synthesis and TNF-α production in the same IgE-dependent PCA reactions (Wershil et al 1995), indicating a possible mechanism of action (by suppression of cytokine cascades) for these agents in the treatment of allergic disease.

In studies of immune complex-mediated inflammation using the peritoneal reverse passive Arthus reaction, mast cells were responsible for the first part of the observed biphasic peak of TNF-α production (Zhang, Ramos and Jakschik 1992a), this initial phase of mast cell activation being due to complement (Ramos, Zhang and Jakschik 1994). Thus, mast cell-derived TNF-α has a role to play in IgE-dependent, IgE-independent and immune complex-mediated inflammation, all of which can occur in skin.

TNF-α also evokes microvascular leakage that can be neutrophil-dependent (Yi and Ulich 1992) or neutrophil- and mast cell-independent (Anderson et al 1994). TNF-α can therefore contribute directly to the vascular, as well as the cellular, components of cutaneous inflammation.

1.8.2.5.2. IL-8

IL-8 is a pro-inflammatory peptide of the "C-X-C" chemokine (chemoattractant cytokine) family, produced abundantly by endothelial cells, fibroblasts and keratinocytes when activated by primary cytokines (IL-1α or β, TNF-α). IL-8 binds to lumenal endothelial surface glycosaminoglycans and activates leukocytes rolling on endothelial selectins (see above; reviewed in Schall and Bacon 1994), it may, therefore, regulate the cellular infiltrate in chronic inflammatory dermatoses (Schröder 1995). A human mast cell leukaemia line (HMC-1) was shown to generate various chemokines including IL-8 (Selvan, Butterfield and Krangel 1994), with exogenous IL-4 increasing IL-8 gene expression in the same cell line (Buckley et al 1995). If this occurs *in vivo*, it would provide a mechanism for mast cell amplification of the inflammatory response. Human mast cells (including those from skin) could not be activated to release histamine by IL-8, unlike human basophils (Füreder et al 1995b). Recombinant human IL-8 (rHu.IL-8) acts as an attractant *in vitro* for neutrophils of various species (Rot 1991), and specifically for those of the sheep (Mulder and Colditz 1993).

Intradermal injection of IL-8 in human skin evokes a neutrophilic infiltrate, in the absence of lymphocytes (Swensson et al 1991). No weal response nor mast cell degranulation was observed (Swensson et al 1991). Initial experiments with intradermal injections of neutrophil-activating peptide-1 (NAP-1; IL-8) in the sheep demonstrated that rapid neutrophil influx ensued (the rate of influx being maximal in the first 30 minutes, and detectable for up to 8 hours), with concomitant plasma leakage of shorter duration (ceased by 6 hours; Colditz, Zwahlen and Bagglioni 1990). Further studies with rHu.IL-8 in ovine skin confirmed the presence of a marked neutrophilic infiltrate with few lymphocytes (Colditz and Watson 1992). Intradermal challenge with recombinant ovine IL-8 in ovine skin gave similar results, with a marked neutrophilic infiltrate also being accompanied by small numbers of eosinophils and increased numbers of $CD4^+$ cells (Seow et al 1994). Assuming that ovine dermal mast cells prove to be capable of IL-8 generation (similar to the HMC-1 cell line) then mast cell-derived IL-8 could play a major role in ovine cutaneous responses characterized by a markedly neutrophilic dermal infiltrate.

1.8.2.5.3. Mast cell-derived cytokine co-ordination in cutaneous inflammation.

In addition to the comparatively immediate effects of some of the mediators described above (histamine, prostaglandins and leukotrienes, proteoglycans, chymases) on vascular permeability, endothelial cell activation and inflammatory cell influx into the dermis, there is probably a longer term, co-ordinated control of the resulting cytokine cascade. This will not only involve mast cell-derived cytokines (whose contribution will be relatively minor in terms of overall cytokine production in skin) but cytokines produced as a consequence of activation of other cell types. Given that relatively little is known concerning the kinetics of mast cell-derived cytokine production in skin, the following is a brief and largely speculative review, predominantly based on *in vitro* findings from cultured mast cell populations. Immediately after mast cell activation, markedly pro-inflammatory cytokines (particularly

TNF-α, but also IL-1, IL-6 and the members of the chemokine family) will rapidly activate endothelial cells and cause increased vascular permeability and emigration of leukocytes to the site of inflammation. The mRNA for this group of cytokines increases within 30 to 60 minutes after IgE-dependent activation (Burd et al 1990, Gordon and Galli 1990a), this response in the case of TNF-α receding within 4 hours of activation (Gordon and Galli 1990a). In the later stages of inflammation, late-generated cytokines mediating autocrine or paracrine events such as IL-13 (reducing the inflammatory activity of TNF-α and IL-1), TGF-β (to stimulate collagen deposition with the development of fibrosis and to act as a mast cell attractant), IL-5 (to recruit and activate eosinophils), IL-3, IL-4 and GM-CSF (to alter the proliferation or function of cells recruited to the site, including mast cells) become of greater importance in the inhibition and resolution of the inflammatory response. For example, different combinations of cytokines are believed to support the four phases of cellular trafficking in the skin [initiation, vasoinduction, effector and resolution](Walsh, Lavker and Murphy 1990). Supporting the above concept of late-generated cytokines, in IgE-mediated BMMC activation increased levels of mRNA for IL-3, IL-5 and GM-CSF did not appear until comparatively late in the response (~120 minutes; Burd et al 1990, Gordon and Galli 1990a). As well as the co-ordinated timing of the cytokine cascade, the nature of the inflammatory response and subsequent local microenvironment may dictate the mast cell-derived cytokines produced. In active human psoriasis for example, it is speculated that dermal mast cells contain IFN-γ, this being released upon activation to stimulate the development of T_{H1}-cells and to induce ICAM-1 on keratinocytes, thereby potentiating the dermatosis (Harvima et al 1994). In contrast, in human cutaneous atopic disease it is speculated that mast cells contain and release IL-4 upon activation, favouring the development a T_{H2}-cell subset and further

amplification of the response (Harvima et al 1994). Thus, the local microenvironment (e.g. psoriatic versus atopic skin) could dictate the pattern of mast cell cytokine response.

1.8.3. Experimental Models Of Mast Cell Involvement In Cutaneous Pathology *In Vivo*

The evidence for the possible contribution of individual mast cell mediators to cutaneous responses has been outlined above. To further support the contention that dermal mast cells can initiate or modulate cutaneous inflammatory responses a brief discussion of cutaneous diseases (selected from both animal models and clinical conditions) in which mast cell involvement has been defined is necessary. Mast cells, and their involvement in both acute and chronic (or late phase) cutaneous responses have been the subject of several recent reviews (Charlesworth 1994, Harvima et al 1994, Marshall and Bienenstock 1994).

The use of the W/W^v mouse model (which has defective *c-kit* receptor production), that allows local reconstitution of dermal mast cells can be achieved by intradermal injection of CM-BMMC obtained from congenic normal (+/+) mice, enables the contribution of the dermal mast cell in cutaneous responses to be elegantly assessed (reviewed in Galli, Tsai and Wershil 1993). W/W^v mice were initially shown to possess a defect in resistance to *Haemaphysalis longicornis* ticks, which could be rescued by either bone marrow or skin transplantation from the congenic normal (+/+) mice (Matsuda et al 1985). This implied that mast cells were important in tick resistance. Further studies using locally dermal mast cell-reconstituted W/W^v mice demonstrated that resistance was conferred only at sites where the local deficiency of dermal mast cells had been repaired (Matsuda et al 1987) and that both IgE antibodies and mast cells were essential for tick resistance (Matsuda et al 1990). The mast cell was shown to play a crucial role in murine tick resistance, this possibly having particular relevance to the sheep, where ectoparasite infestation is common. Idiopathic chronic dermatitis occurs spontaneously in both W/W^v and Sl/Sl^d mice, this resulting in the

local development of mature mast cells at the site of dermatitis lesions in the former but not the latter (Galli et al 1987), possibly indicating microenvironmental generation of growth factors. Certainly, mast cell hyperplasia has been associated with dermatitis of varying aetiologies (Yamamoto et al 1995). Using phorbol 12-myristate 13-acetate (PMA) as a non-immunologic mast cell activator in *Sl/Sl*^{d}, *W/W*^{v}, locally mast cell reconstituted *W/W*^{v} and congenic normal (+/+) mice, it was demonstrated that the presence of mast cells increased both the tissue swelling and influx of leukocytes into the dermis, thereby amplifying this non-immunologic response (Wershil, Murakami and Galli 1988). Repeated cutaneous application of PMA results in the development of both a dermatitis and a significant increase in dermal mast cell numbers in both *W/W*^{v} and congenic normal (+/+) mice, these mast cells being able to express an IgE-dependent PCA response (Gordon and Galli 1990b). The deposition of fibrin in IgE-dependent immediate hypersensitivity reactions using *W/W*^{v} and locally dermal mast cell-reconstituted *W/W*^{v} mice was shown to be mast cell-dependent (Wershil et al 1987), this study also proving that mast cells are required for PCA reactions. In the same model, where IgE-dependent and contact sensitivity (using oxazolone) reactions performed simultaneously at the same site were compared in terms of fibrin deposition against the same two reactions occurring separately at different sites, the effect of IgE-dependent mast cell activation on the contact sensitivity response was to increase fibrin deposition by 3 to 25 times between 2 and 6 hours post-challenge (Mekori and Galli 1990). There was no effect of mast cell deficiency on the ability to express contact sensitivity responses, however fibrin deposition was noted as soon as two hours after initiation of the response (Mekori and Galli 1990). Therefore, mast cells were shown to be necessary for the IgE-mediated augmentation of fibrin deposition in contact sensitivity responses, but not for the fibrin deposition in contact sensitivity responses themselves (Mekori and Galli 1990). In studies on the role of sP in neurogenic inflammation using *W/W*^{v} and *Sl/Sl*^{d} mice, the increase in vascular permeability

and neutrophil infiltration was shown to be predominantly mast cell-dependent (Yano et al 1989). The dermal mast cell was also shown to enhance the inflammatory process in cutaneous immune-complex-induced injury (reverse Arthus reaction), local reconstitution of mast cells in W/W^v mice increasing neutrophil emigration and oedema to the same level as in +/+ controls (Zhang, Ramos and Jakschik 1991). Additionally, the contribution of the dermal mast cell and mast cell-derived histamine and leukotrienes to immune-mediated basement membrane damage in skin was ascertained (Zhang, Ramos and Jakschik 1992b). The mast cell-deficient mouse model was also used to show that the subcutaneous injection of rrSCF into Sl/Sl^d or +/+ congenic normal mice induced the development of CTMC, in part due to the proliferation of differentiated dermal mast cells (Tsai et al 1991b).

Thus, the use of W/W^v or Sl/Sl^d mast cell-deficient mice has allowed the contribution of the dermal mast cell to be ascertained in various cutaneous responses (i. Non-immunologic, ii. IgE-mediated, iii. Contact sensitivity, iv. Neuropeptide-induced, v. Immune complex-mediated).

1.9. Aims Of The Study

Comparatively little is known specifically about the ovine dermal mast cell and ovine mast cell heterogeneity, although from the above there is ample *in vitro* and *in vivo* evidence to implicate mast cells and mast cell-derived mediators in the pathogenesis of cutaneous inflammation. Furthermore, few studies have addressed the question of the role of direct mast cell activation on the alteration of dermal inflammatory cell trafficking *in vivo*. The aims of the study were therefore:-

1) To determine if mast cell heterogeneity, as defined in other species by differences between tissues in the distribution of mast cell chymase, existed in the sheep.

2) To characterize and define, using secretagogues known to activate cutaneous mast cells in other species, the inflammatory responses and changes in dermal inflammatory cell trafficking evoked by these agents in the sheep *in vivo*.

3) To determine if the ovine chymase SMCP could evoke an inflammatory response when injected into the skin of the homologous host *in vivo* and, if so, to characterize this cutaneous response.

4) By cannulating afferent lymphatics in flank skin, to monitor the changes in inflammatory cell trafficking through the dermis after direct mast cell activation with the secretagogues defined in (2) above and, similarly, following injection of SMCP (Discussed in Chapter 8).

5) To characterize the *in vitro* kinetics of development of rOv.IL-3 BMMC, their mediator content and likely heterogeneity.

6) To determine the functional activity of optimally cultured rOv.IL-3 BMMC in response to activation by secretagogues defined in (2) above and to SMCP.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

All chemicals, (unless indicated otherwise) were purchased from either BDH Ltd., Poole, Dorset, UK. ('AnalaR' [analytical reagent] or GPR [general purpose reagent] grades), Sigma Chemical Company Ltd., Poole, UK or Fisons Scientific Equipment, Loughborough, UK.

2.2. Animals

All sheep were bred at the Moredun Research Institute, Gilmerton Road, Edinburgh and were either Blackface, Suffolk-cross or Finn-Dorset crosses. For tissue culture studies animals were between four and nine months old, whereas cast ewes were used in all other studies. All experiments involving the use of animals conformed to ethical guidelines and were peer reviewed by committee (including statistical assessment of the study protocol) before being undertaken. All sheep to be housed were treated with a topical ectoparasiticide to remove lice and other ectoparasites (deltamethtrin 1% $^{w}/_{v}$, Spot On Insecticide, Coopers®; Pitman-Moore Ltd., Crewe, UK) four weeks before being housed, and once while housed.

2.3. Ovine Bone Marrow Cultures

2.3.1. Ovine Bone Marrow Cell Culture Set-up

Immediately post mortem, the sternum of a four to nine month old lamb was removed, sprayed with 70% alcohol and the attached fat and muscle removed. After again spraying with alcohol, the sternum is placed in a vice, and sawn longitudinally (manubrium to xiphoid). The half-sternum is clamped in the vice (cut surface uppermost), pressure applied, and the bone marrow expressed. Using a sterile scalpel blade or sterile 5ml syringe (Plastipak, Becton Dickinson, Oxford, UK, Cat.No. 302187) with a sterile 19G needle (Microlance®, Becton Dickinson, Oxford, UK, Cat.No. 001500110) the bone marrow is collected and placed into a universal containing Hanks' Balanced Salt Solution (HBSS)(Gibco BRL, Life Technologies

Ltd., Paisley, UK, Cat.No. 041-04020M) to which penicillin, streptomycin and heparin has been added (HBSS/heparin medium, Appendix A). The closed universal is sprayed with alcohol, transferred to sterile conditions in a tissue culture hood (ICN Flow Gelaire® Class II BSB48, ICN Pharmaceuticals Ltd., Thame, UK) and allowed to settle. The cell suspension from below the fat layer is removed and passed through a double layer of sterile lens tissue into a clean universal. The cell suspension is then spun at 1300 r.p.m. in a Beckman® Model TJ-6 centrifuge at 4 °C for five minutes. Six universals are prepared, each containing 8ml of Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway, Cat.No. 1001967). The cell pellet is resuspended in 24ml HBSS/heparin, and this suspension divided equally between the six Lymphoprep™ universals. The cell suspension is carefully layered onto the Lymphoprep™ using a 5ml sterile disposable pipette (Sterilin, Bibby Sterilin Ltd., Stone, UK, Cat.No. 03122). Without agitation, the universals are placed in the centrifuge and spun at 1800-2000 r.p.m. for 30 minutes at 4 °C. The interface cells are collected using a sterile syringe and needle, and placed in a 50ml centrifuge tube (Costar UK Ltd., High Wycombe, UK, Cat.No. 6751). HBSS/heparin is added to a total volume of 50ml to wash the cells, and the suspension centrifuged at 1300 r.p.m. for five minutes at 4 °C. This washing procedure is repeated twice. The final pellet is resuspended in Iscove's Modification of Dulbecco's Medium (IMDM) to which heat-inactivated foetal calf serum, penicillin and streptomycin has been added (IMDM/10% FCS; Appendix A). A viable cell count is undertaken and the cells plated out at 2×10^5 cells/ml of tissue culture medium in either sterile, disposable 24 well tissue culture plates (24 Well Cell Culture Cluster, Costar UK Ltd., High Wycombe, UK, Cat.No. 3524) or flasks ($75cm^2$ canted neck flasks, Corning Inc., New York, USA, Cat.No. 25110-75). Recombinant ovine interleukin-3 (rOv.IL-3), a generous gift from Dr.G. Entrican (Moredun Research Institute, Edinburgh), was used to supplement the IMDM/10%FCS. This had been expressed in a Chinese Hamster Ovary (CHO) cell expression system (M^{c}Innes, Haig and

Logan 1993), the rOv.IL-3 being in the form of a tissue culture supernatant. In the case of experiments where 24 well tissue culture plates were used, the batch of rOv.IL-3 used was 3/2/4/TF/3/A/19/2/10; 750µM MSX + FBS 1/8/94. The inclusion ratio of rOv.IL-3 was expressed as the final dilution of this original supernatant in each tissue culture well or plate. Cells were maintained in an atmosphere of 5% CO_2/air at a temperature of 37 °C in a tissue culture incubator (ICN Flow Automatic CO_2 Incubator Model 160, ICN Pharmaceuticals Ltd., Thame, UK). Cells were generally fed every five to seven days. For 24 well plates this involved removal of a known volume of supernatant without agitating the plate, and replacement of this with an equivalent volume of fresh medium. The progress of the culture was routinely monitored by daily examination under a Leica DM 1L microscope (Leica Microscopy, Germany, Cat.No. 520802/152192), and by microscopic examination of Leishman's and/or anti-SMCP stained cytosmear preparations.

2.3.2. Harvesting Procedure For Cell Populations In 24 Well Plates

Without disturbing the plate, 100µl of cell supernatant was removed from the well, placed in an eppendorf and stored at -70 °C until assayed. The well was then gently agitated to produce a uniform cell suspension. This was removed and placed in an eppendorf tube, the total volume of cell suspension being noted. 250µl of cell suspension was removed and used to make two 125µl cytosmears (one for Leishman's staining and one fixed for anti-SMCP immunohistochemistry). A further 15µl of cell suspension was removed and a viable cell count performed. The remaining cell suspension was centrifuged for 5 minutes (Anderman Eppendorf centrifuge 5414, Germany) at 12000 r.p.m. The supernatant was discarded, the cell pellet being stored at -70 °C until being assayed.

2.3.3. Cell Counts

Cell viability was assessed by nigrosin dye exclusion. Nigrosin (0.1% nigrosin in PBS; Nigrosin [Acid Black 2, Cat.No. N4754] from Sigma Chemical Company Ltd., Poole, UK) was mixed 1:1 with the cell suspension and left for five minutes before being counted. Using an improved Neubauer haemocytometer, the number of viable and non-viable cells in four large squares (each comprising 16 smaller squares of total volume 0.1µl) was counted. After correcting for the dilution effect due to the addition of nigrosin/PBS, the result was expressed as the mean number of viable cells per ml of cell suspension.

2.3.4. Cytosmear Preparations

Cytosmear preparations were prepared using a Shandon Cytospin 2 cytocentrifuge (Shandon Southern Products Ltd., Runcorn, UK), 125µl of cell suspension (routinely 5 $x10^5$ cells/ml, but for cell culture studies (Chapter 6) this was dependent on the cell density in the well) being centrifuged at 600 rpm for five minutes. Cytosmears were air-dried before fixation and/or staining.

2.3.5. Leishman's Staining Of Cytosmear Preparations

After air-drying the cytosmear, a sufficient volume of Leishman's staining solution (eosin methylene blue compound 0.2% $^{w}/_{v}$ in methanol, Gurr® microscopy materials, BDH, Poole, UK, Cat.No.35022) to cover the slide was added for two minutes. An equivalent volume of distilled water was subsequently added for a further eight minutes. After rinsing thoroughly in tap water the slides were air-dried and mounted using CoverBond™ (American Scientific Products, American Hospital Supply Corporation, Illinois, USA, Cat.No. M7639-16) mountant.

2.3.6. Fixation Of Cytosmear Preparations For Immunohistochemistry

After air-drying, cytosmear preparations were fixed in 4% paraformaldehyde (PF; Fisons Scientific Equipment, Loughborough, UK, Cat.No. P/0840/53)/PBS pH 7.4 (Appendix A) for 1 hour at 45 °C. Fixed preparations were then stored in 70% ethanol at 4 °C until use.

2.3.7 Affinity Purification Of Rabbit Anti-Sheep Mast Cell Proteinase (rabαSMCP) Polyclonal Antibody From High Titre Serum

Initially an IgG fraction was prepared from high titre anti-SMCP rabbit serum, using a Protein A-Sepharose column (Pharmacia Biotech Ltd., Milton Keynes, UK, Cat.No. 17-0403-01) and a Pharmacia® FPLC system. This was to ensure that inhibitors of SMCP (α_2-macroglobulin or rabbit serpins [serine proteinase inhibitors]) were removed prior to the affinity chromatography step. The Protein A column was initially equilibrated with PBS/0.01% sodium azide running buffer, pH 7.5. 2ml of serum was applied to the column, the immediate flow-through being discarded. The bound IgG fraction was subsequently eluted with 0.1M citric acid/0.5M sodium chloride pH 2.5 elution buffer. The eluate was titrated to pH 7.0-7.5 with 1M Tris. The Protein A-Sepharose column was again equilibrated with running buffer, and a further two runs with high-titre serum undertaken.

SMCP had been previously bound to cyanogen bromide-activated sepharose (CNBr-activated Sepharose 4B, Pharmacia Biotech Ltd., Milton Keynes, UK, Cat.No. 17-0430-01), to produce an SMCP affinity column. Once equilibrated with running buffer (see above), the eluted IgG fraction from the protein A column was applied in aliquots, and the flow-through discarded. The bound anti-SMCP antibody was eluted using elution buffer (see above) and the pH was adjusted to pH 7.0-7.5 with 1M Tris. The affinity column was equilibrated with running buffer and the cycle was repeated until all of the eluted IgG fraction had been affinity purified. The affinity purified rabαSMCP antibody was concentrated using a collodion bag (Sartorius Ltd., Epsom, UK, Cat.No. SM 13200) and negative pressure. Once concentrated, a Pierce

assay (BCA* Protein Assay Reagent, Pierce & Warriner, Chester, UK, Cat.No., 23225) was undertaken to determine the protein concentration of the rabαSMCP, and the affinity purified antibody stored at -70 °C at 1mg protein/ml. From the original 6ml of high titre serum, 2.55mg affinity purified rabαSMCP was obtained (i.e. a yield of 0.425mg rabαSMCP/ml high titre serum). The presence of affinity purified rabαSMCP antibody was confirmed by titrating on SMCP coated microtitre plates in a modified ELISA, and by positive immunohistochemical staining of SMCP on tissue sections of ovine thymus or gut and on cytosmear preparations of ovine bone marrow-derived mast cells grown in the presence of recombinant interleukin-3. The antibody (batch 148/93) was subsequently titrated for use in routine immunohistochemical staining of tissue sections and cytosmear preparations.

(N.B. The monospecificity of rabαSMCP prepared by this method had been previously investigated by Western blotting of samples prepared from homogenates of abomasal tissue, or extracts of isolated mucosal mast cells from *Haemonchus* infected sheep (Huntley 1991). RabαSMCP had been shown to react with a single polypeptide band of MW 25000. No bands had been observed in control blots where rabαSMCP was substituted with normal rabbit immunoglobulin. A monoclonal antibody to SMCP was made available towards the end of the period of these studies, this being shown to have the same degree of specificity as that of the affinity purified polyclonal antibody).

2.3.8. Demonstration Of SMCP In Fixed Cytosmears

2.3.8.1. 3,3′- Diaminobenzidine (DAB) Method

Fixed cytosmears, stored at 4 °C in 70% ethanol, were subjected to the following protocol:-

i. Wash in tap water for 15 minutes.

ii. Treat with 1% aqueous Periodic acid (Periodic Acid GPR, BDH Ltd., Poole, UK, Cat.No.29842 2P) for five minutes.

iii. Wash in tap water for 10 minutes.

iv. Incubate for five minutes with 0.2% Sodium borohydride GPR (BDH Ltd., Poole, UK, Cat.No. 301143N) in 1% disodium hydrogen orthophosphate (Fisons Scientific Equipment, Loughborough, UK, Cat.No.301143N). [Steps (ii) and (iv) are included to block any endogenous peroxidase activity that may be present; Heydermann and Neville (1977)].

v. Wash in tap water for 10 minutes.

vi. Place in 5% Bovine serum albumin (BSA, Sigma Chemical Company Ltd., Poole, UK, Cat.No.A2153 or BSA, protease free, fraction V, ICN Pharmaceuticals Ltd., Thame, UK, Cat.No.810035)/PBS (Appendix A) for 15 minutes at room temperature.

vii. Incubate with affinity-purified rabbit IgG anti-SMCP (2.5µg /ml 5% BSA/PBS)(Huntley et al 1986; batch numbers 90/90 and 148/93) in a humidity chamber for 1 hour at room temperature. Controls consisted of normal rabbit serum (NRS, 1:500 dilution in 5%BSA/PBS) or 5%BSA/PBS in place of rabαSMCP.

viii. Wash three times in PBS.

ix. Incubate in a humidity chamber with goat anti-rabbit IgG-horseradish peroxidase (2.5µg/ml, Sigma Chemical Company Ltd., Poole, UK, Cat.No.A0545; cross-adsorbed against sheep IgG, in 5% BSA/PBS) for 1 hour at room temperature.

x. Wash three times in PBS.

xi. Reveal peroxidase activity with 3,3′-diaminobenzidine (DAB; 3,3′- diaminobenzidine tetrahydrochloride, Sigma Chemical Company Ltd., Poole, UK [Cat.No.D5637]/PBS (Appendix A) or Sigma Fast® tablet sets, Sigma Chemical Company Ltd., Poole, UK [Cat.No.D4293]).

xii. Wash in tap water for five minutes.

xiii. Counterstain with haematoxylin (Ehrlich's haematoxylin original formula, Gurr® microscopy materials, BDH, Poole, UK, Cat.No. 350172Q) for one minute, wash in tap water for five minutes.

xiv. Dehydrate stained cytosmears through graded alcohol, clear in xylene and mount in CoverBond™ (see "Leishman's staining of cytosmear preparations" above).

2.3.8.2. Vectastain ® ABC-Alkaline Phosphatase (ABC-AP) Method

This involved the use of a commercially available biotin/avidin-AP system in conjunction with the rabαSMCP primary antibody. As optimisation of the system for use on cytosmear preparations and tissue sections led to differences between the staining procedure recommended with the Vectastain® ABC-AP kit (Vector Laboratories Inc., Peterborough, UK, Cat.No. AK5001) and that actually used, the method is outlined in full below.

i, ii, iii. These steps are as outlined above (2.3.8.1.).

iv. Incubate cytosmears for five minutes with 0.2% Sodium borohydride GPR (BDH Ltd., Poole, UK, Cat.No.301143N) in 1% disodium hydrogen orthophosphate (Fisons Scientific Equipment, Loughborough, UK, Cat.No.301143N). [As a range of tissue sections including intestine were also stained using this method, steps (iii) and (iv) were included routinely for all preparations. These steps inhibit the intestinal isoenzyme of alkaline phosphatase (Bulman and Heydermann 1981)].

v. Wash in tap water for 10 minutes.

vi. Incubate in normal goat serum (NGS, from kit) at 1:67 dilution in PBS (Appendix A) in a humidity chamber at room temperature for 20 minutes.

vii. Blot excess NGS from cytosmears.

viii. Incubate with affinity-purified rabbit IgG anti-SMCP (2.5µg rabαSMCP/ml PBS)(Huntley et al 1986; batch numbers 90/90 and 148/93) in a humidity chamber overnight at 4 °C. Controls consist of NGS (as above), NRS (1:500 dilution in PBS) or PBS in place of primary antibody.

ix. Wash in PBS for 10 minutes (4 changes of PBS).

x. Incubate with goat biotinylated anti-rabbit IgG (H + L) (from kit, 1 drop to 10ml PBS) in a humidity chamber for 1 hour at room temperature.

xi. Prepare Vectastain® ABC-AP reagent (from kit) and allow to stand for 45 minutes before use (2 drops reagent A plus 2 drops reagent B to 10ml PBS).

xii. Wash cytosmears in PBS for 10 minutes (4 changes of PBS).

xiii. Incubate in a humidity chamber for 60 minutes at room temperature with Vectastain® ABC-AP reagent.

xiv. Wash in PBS for 10 minutes (4 changes of PBS).

xv. Incubate for 30 minutes in alkaline phosphatase substrate (Vector® Red, [Alkaline phosphatase kit I], Vector Laboratories Inc., Peterborough, UK, Cat.No. SK-5100) to which levamisole has initially been added (1 drop of 125mM levamisole [Levamisole solution, Vector Laboratories Inc., Peterborough, UK, Cat.No.SP-5000] to 5ml 100mM

Tris HCl substrate buffer, pH 8.2). (The addition of levamisole inhibits isoenzymes of AP other than the intestinal form).

xvi. Wash in tap water for 15 minutes.

xvii. Counterstain with haematoxylin (Ehrlich's haematoxylin original formula, Gurr® microscopy materials, BDH, Poole, UK, Cat.No. 350172Q) for one minute and wash in tap water for five minutes.

xviii. Dehydrate stained cytosmears through graded alcohol series, clear in xylene and mount in CoverBond™.

2.3.9. Toluidine Blue Staining Of Fixed Cytosmears

Fixed cytosmears, stored in alcohol at 4 °C, were washed in tap water for ten minutes. They were then placed in 0.5% toluidine blue (Toluidine blue 0, Sigma Chemical Company Ltd., Poole, UK, Cat.No. T3260) in 0.5N HCl pH 0.5 for 30 minutes (Enerback 1966a) before washing in water and dehydrating through a graded alcohol series. Cytosmears were cleared in xylene and mounted in CoverBond™.

2.3.10. Enumeration Of Mast Cells In Anti-SMCP Stained Cytosmear Preparations

Cytosmear preparations were routinely stained to demonstrate the presence of SMCP by the Vectastain® ABC-AP method previously described. Using a Leitz Wetzler Dialux 20 EB microscope at 500x magnification the number of mast cells identified in a random sample of 200 cells in each cytosmear was noted (based on positive staining for SMCP and compatible nuclear morphology). The percentage of mast cells present in the cytosmear preparation could thus be calculated.

2.3.11. Extraction Of Cell Pellets For Assay Of SMCP Content, Arylsulfatase And β-hexosaminidase Activity

This has been previously described by Huntley et al (1992). Cell pellets, stored at -70 °C, were thawed and 200µl of extraction buffer (20mM Tris/HCl buffer pH 7.5 containing 2M KCl) added. The cell suspension was freeze-thawed rapidly three times with vortex-mixing between cycles, and centrifuged for two minutes at 12000 r.p.m. (Anderman Eppendorf centrifuge 5414, Germany). The supernatant was removed and assayed for the appropriate mediator.

2.3.12. ELISA For Sheep Mast Cell Proteinase (SMCP)

For all initial studies, the SMCP ELISA utilised polyclonal antibodies for capture and detection of SMCP (method A below). Towards the end of these studies monoclonal antibodies to SMCP were produced in the laboratory, and thus a polyclonal/monoclonal antibody ELISA was developed (method B below). All measurements concerning release of SMCP from rOv.IL-3 derived BMMC were determined using the latter method.

2.3.12.1. Method A : Polyclonal Antibody Based SMCP ELISA

i. Coat 96 well microtitre ELISA plates (M129B [of German origin], Dynatech Laboratories Ltd., Billingshurst, UK) with 50µl per well of affinity-purified rabbit IgG anti-SMCP capture antibody (Batch 114/91) at 1µg/ml in 0.1M carbonate ELISA coating buffer, pH 9.6 (Appendix A) overnight at 4 °C.

ii. Wash plates six times with PBS/0.05% Tween 20 (Poloxyethylenesorbitan monolaurate, Sigma Chemical Company Ltd., Poole, UK, Cat.No. P1379).

iii. Load SMCP standards (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 ng SMCP/ml) or samples made up in PBS/0.05% Tween 20 in duplicate onto the coated plate (50µl of standard or sample per well). Samples were assayed at a range of dilutions (normally 1:10

to 1:10000) in order that the OD_{492nm} reading of the sample would lie (for at least one of the dilutions) on the linear portion of the standard curve. Controls consist of 50µl PBS/0.05% Tween 20 alone. Incubate plate for 1 hour at room temperature.

iv. Wash plate six times with PBS/0.05% Tween 20.

v. Add 50µl of a 1:2000 dilution of rabαSMCP-horseradish peroxidase conjugate (Batch No. 105/91; prepared by G.Newlands, Moredun Research Institute, Edinburgh - conjugation method as per Nakane and Kawaoi (1974)) in PBS/0.05% Tween 20 to each well, and incubate the plate for 1 hour at room temperature.

vi. Wash plates six times with PBS/0.05% Tween 20.

vii. Colour reaction was developed by the addition of 50µl per well of orthophenylenediamine (OPD) in citrate/phosphate buffer, pH 5.0 (Appendix A) or of substrate produced from SigmaFast™ OPD tablet sets (Sigma Chemical Company Ltd., Poole, UK, Cat.No. P9187).

viii. Stop colour development by the addition of 25µl of 2.5M H_2SO_4 per well, and read plates at OD_{492nm} in a Titertek® Multiskan MC Reader (Titertek® Flow Laboratories).

A standard curve produced from the SMCP standard set was plotted using Dynatech data analysis software (Dynatech Laboratories Ltd., Billingshurst, UK), and the concentration of SMCP in samples calculated from the linear portion of the standard curve. A sample standard curve for the SMCP ELISA is shown in Fig.2.1. Results were expressed as ng of SMCP per cell pellet, as ng SMCP per cell population of the tissue culture well, or as ng SMCP per ml tissue culture well supernatant. (For assay of tissue samples [see later] results were expressed as µg SMCP per g wet weight of tissue).

2.3.12.2. Method B : Polyclonal/Monoclonal Antibody Based SMCP ELISA

i, ii, iii, iv. These steps are as outlined above (2.3.12.1.).

v. Add 50µl of a 1:500 dilution of biotinylated monoclonal ratαSMCP antibody (Batch No. 165/95) in PBS/0.05% Tween 20 to each well and incubate the plate for 1 hour at room temperature.

vi. Wash plates six times with PBS/0.05% Tween 20.

vii. Add 50µl of a 1:4000 dilution of Streptavidin-peroxidase conjugate (Streptavidin-POD, Boehringer Mannheim UK, Lewes, UK, Cat.No. 1089153) to each well, and incubate the plate for 30 minutes at room temperature.

viii. Wash plates six times with PBS/0.05% Tween 20.

Add 50µl per well of substrate produced from SigmaFast™ OPD tablet sets (Sigma Chemical Company Ltd., Poole, UK, Cat.No. P9187). Stop colour development by the addition of 25µl of 2.5M H_2SO_4 per well and read the plates at OD_{492nm} in a Titertek® Multiskan MC Reader (Titertek® Flow Laboratories). Sample SMCP concentrations were calculated as described in method A.

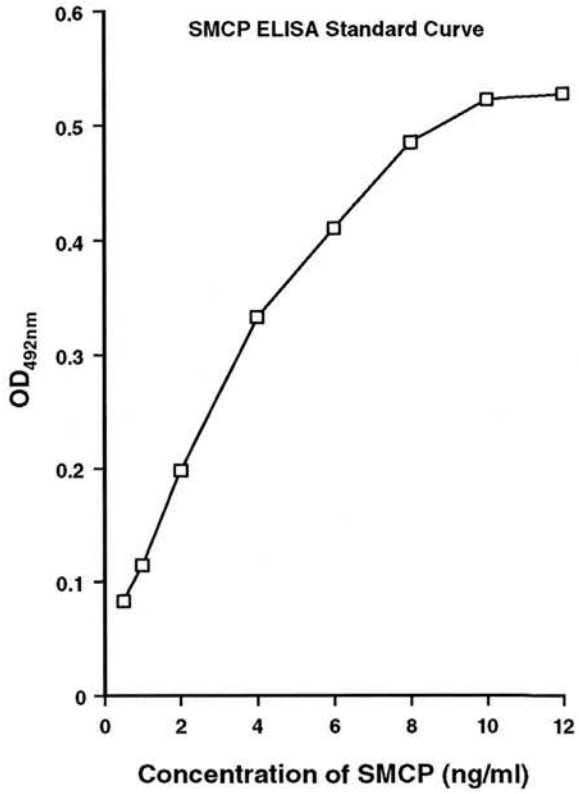


Figure 2.1. Representative example of an SMCP ELISA Standard curve. In this case only the first four datapoints lie on the linear portion of the curve.

2.3.13. Assay Of Arylsulfatase Activity

i. Add 20µl of sample or standards (Sulfatase type V, Sigma Chemical Company Ltd., Poole, UK, Cat.No. S 8629; 1.95µg arylsulfatase/ml to 250µg arylsulfatase/ml) to 80µl substrate (6.255mM p-nitrocatechol sulphate [Sigma Chemical Company Ltd., Poole, UK, Cat.No. N7251] in 0.2M acetate buffer, pH 5.7, Appendix A) in a microtitre plate.

ii. Incubate the plate for 60 minutes at 37 °C and terminate the reaction by the addition of 100µl of 5M NaOH. The plate is read at OD_{492nm} in a Titertek® Multiskan MC Reader (Titertek® Flow Laboratories).

iii. From the arylsulfatase standard readings a standard curve is plotted, and using FigP software (Fig.P Corp®, Biosoft®, Cambridge, UK) a regression line calculated. The activity of arylsulfatase in samples was calculated from

sample arylsulfatase activity = (sample OD_{492nm} - c)/ m

where m = gradient of regression line and c = regression line constant. A sample standard curve is shown in Figure 2.2. below.

iv. Arylsulfatase activity in unknown samples derived from the standard curve was expressed as units of arylsulfatase activity (u) per cell population of each tissue culture well or for supernatants as units of activity (u) per total well tissue culture supernatant.

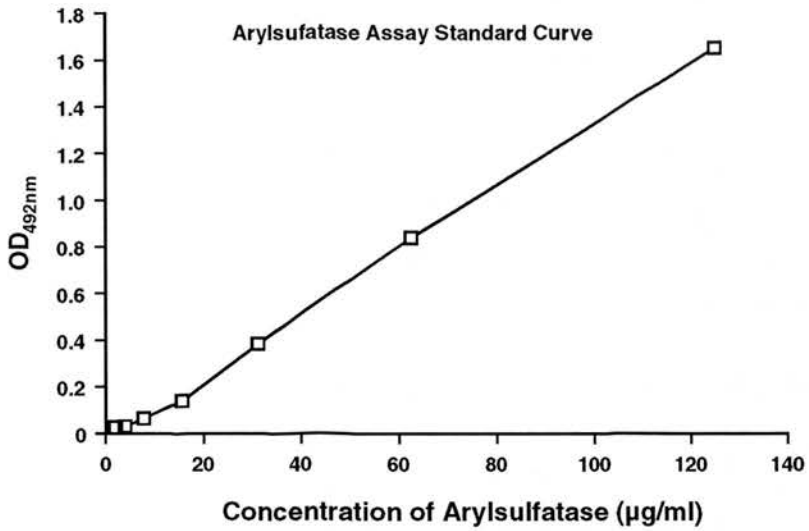


Figure 2.2. Representative standard curve from arylsulfatase assay.

2.3.14. Assay Of β-Hexosaminidase Activity

β-hexosaminidase activity was assayed by hydrolysis of the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Company Ltd., Poole, UK, Cat.No.N9376); 1 unit of

enzyme cleaving 1µmole of substrate per hour at 37 °C (Schwartz, Austen and Wasserman 1979). 25µl of sample is added to 50µl of substrate solution (5mM substrate in citrate buffer, pH 4.5; Appendix A) for 45 minutes at 37 °C. The reaction is stopped by the addition of 200µl of ice-cold glycine NaOH buffer, pH 10.7 (Appendix A) and the plate read at OD_{405nm} in a Titertek® Multiskan MC Reader (Titertek® Flow Laboratories). The OD_{405nm} for controls (where buffer had been used in place of sample) were subtracted from the OD_{405nm} for samples and sample β-hexosaminidase activity calculated using $E=18.8x10^3$l/mol/cm at OD_{405nm}. Using the experimental set up described above, the activity was calculated from the formula:-

β-hexosaminidase activity (units) =

$(\Delta OD_{405nm}$ at 45 min)/(0.8 x $18.8x10^3$) x (TRV/TSV) x 1.2735

(where TRV is the total reaction volume and TSV the total sample volume).

The result was expressed as units of β-hexosaminidase activity per cell population of the tissue culture well or for supernatants as units of activity per total well tissue culture supernatant.

2.3.15. rOv.IL-3-Dependent BMMC Mediator Release Studies

Bone marrow cells grown in flasks in the presence of an optimal dilution of rOv.IL-3 were centrifuged at 800 r.p.m. for five minutes at 4 °C (N.B. Release studies were performed only when cultures contained 40-50% mast cells assessed by examination of Leishman's stained cytosmear preparations). The supernatant was discarded, and the cell pellet resuspended in 1x Earle's medium (Gibco BRL, Life Technologies Ltd., Paisley, UK, Cat.No. 042-04050H). The 'washed' cell suspension was centrifuged at 800 r.p.m. for five minutes at 4 °C, the supernatant discarded and the cell pellet resuspended in Earle's medium. A cell count was

undertaken, and cytosmear preparations of the cell suspension made for both Leishman's staining and fixed for anti-SMCP immunohistochemistry. Aliquots of cell suspension equivalent to 0.5 or 1 $x10^6$ cells were placed in sterile eppendorfs. The putative release agent (sP, 48/80, A23187, SMCP or HI-SMCP) was added at a range of concentrations to give a final total volume of 200µl. As controls, the diluent in which each agent had been prepared was added to a second series of pellets across the same range of concentrations, again giving a final volume of 200µl. Additional controls consisted of the addition of Earle's medium alone in place of agent. After incubation at 37 °C for 45 minutes (with gentle mixing) the eppendorfs were centrifuged at 12000 r.p.m. for 5 minutes at 4 °C. The supernatants were removed and stored at -70 °C for subsequent assay of mediator content or activity. To assess the total mediator content or activity of the cell pellet, separate control, non-stimulated pellets were subjected to the addition of 200µl of 20mM Tris/HCl pH 7.5 containing 2M KCl, the cell suspension being freeze-thawed rapidly three times with vortex-mixing between cycles, and centrifuged at 12000 r.p.m. for five minutes at 4 °C. The extracted pellet supernatant was removed and assayed for its SMCP, arylsulfatase and β-hexosaminidase content. All measurements were undertaken on duplicate pellets, and in a minimum of two separate cultures. The percentage of mediator release for test and diluent-treated samples was expressed as a percentage of the total content or activity of mediator assayed after extraction from separate duplicate, non-stimulated control cell pellets. i.e.:-

Percentage release

= {(quantity of mediator in secretagogue- or diluent-treated pellet supernatant)/

(quantity of mediator in extracted pellet)} x 100

2.3.16. Transmission Electron Microscopy Of rOv.IL-3 BMMC

This was undertaken with the generous help of Mr. S.Mitchell, Electron Microscope Unit, RDSVS. Samples were processed as per the following protocol:-

i. Fix samples in 3% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 hours.

ii. Wash samples in 0.1M sodium cacodylate buffer (three cycles of 20 minutes).

iii. Post-fix in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 60 minutes.

iv. Wash in distilled water (three cycles of 20 minutes).

v. Dehydrate through graded (50%, 70%, 90%, 100%) acetone series with single ten minute cycles, except for 100% acetone with three cycles of ten minutes.

vi. Infiltrate sample with 50:50 araldite:acetone mix (mixed for 30 minutes prior to use) overnight in 60°C oven. Then place sample through three 60 minute cycles using fresh 50:50 araldite:acetone mix. To complete sample infiltration use araldite mix with accelerator through two 60 minute cycles.

vii. Embed sample in araldite mix with accelerator for 48 hours in a 60°C oven.

viii. Cut 60nm sections on a Reichert OMU4 Ultracut® ultramicrotome using a diamond knife and mount on 200 mesh copper grids.

ix. Stain sections with uranyl acetate and lead citrate using an LKB Ultrostainer®.

x. View and photograph sections using a Philips 400® transmission electron microscope.

All processing must be undertaken in a fume-chamber, with constant rotation of specimens except during the overnight infiltration (**vi**) and embedding (**vii**).

2.4. Ovine Mast Cell Heterogeneity Studies

2.4.1. Tissue Fixation For Histochemistry and Immunohistochemistry

Tissue was fixed in 4%PF/PBS (Appendix A) for six hours at room temperature (Newlands et al 1984). Fixed samples were transferred to 70% ethanol and stored at 4 °C until processing (Miller et al 1983), when they were embedded in paraffin wax.

2.4.2. Histochemical And Immunohistochemical Staining Of Paraffin Embedded Tissue

Sections, usually cut at 4μm, were deparaffinized in xylene, rehydrated through a graded alcohol series to water, and stained by the appropriate stain as described below.

2.4.2.1. Toluidine Blue Stain For Mast Cells

The method is as described under "Toluidine blue staining of fixed cytosmears".

2.4.2.2. Carbol Chromotrope (Lendrums) Stain For Eosinophils

i. Stain tissue sections with haematoxylin (Ehrlich's haematoxylin original formula, Gurr® microscopy materials, BDH, Poole, UK, Cat.No. 350172Q) for one minute.

ii. Wash in tap water for five minutes.

iii. Stain in carbol chromotrope (0.5% chromotrope in 1% phenol, Appendix A) for 30 minutes.

iv. Wash in tap water for five minutes.

2.4.2.3. Haematoxylin and Eosin Stain

i. Stain tissue sections with haematoxylin (Ehrlich's haematoxylin original formula, Gurr® microscopy materials, BDH, Poole, UK, Cat.No. 350172Q) for one minute.

ii. Wash briefly in Scotts tap water substitute and then in tap water for five minutes.

iii. Counterstain in Eosin (1% aqueous solution).

iv. Wash in tap water for five minutes.

2.4.2.4. Demonstration Of SMCP

2.4.2.4.1. 3,3'- Diaminobenzidine (DAB) method

The procedure used is as described under "Demonstration of SMCP in fixed cytosmears" (2.3.8.1.) from steps **ii** to **xiii**.

2.4.2.4.2. Vectastain® ABC-alkaline phosphatase (ABC-AP) method

The method used was as described under "Demonstration of SMCP in fixed cytosmears" (2.3.8.2.) from steps **ii** to **xvii** except that slides were coated with a tissue section adhesive (Vectabond™, Vector Laboratories Inc., Peterborough, UK, Cat.No. SP-1800) prior to processing. In addition, in initial experiments the tissue section was incubated with the rabαSMCP primary antibody for 30 minutes in a humidity chamber at room temperature. Optimisation of the staining procedure for cytosmear preparations indicated that incubation overnight in a humidity chamber at 4 °C gave greater intensity of staining, this protocol being adopted in later experiments.

Stained tissue sections were subsequently dehydrated through a graded alcohol series, cleared in xylene and mounted in CoverBond™.

2.4.3. Preparation Of Tissue Samples For Assay By SMCP ELISA

Universal containers were pre-weighed, labelled, and held on ice. Immediately post mortem tissue samples were collected, weighed, and stored at -70 °C until assay. After thawing, five volumes of ice cold 20mM Tris/HCl buffer pH 7.5 containing 1.5M NaCl were added per tissue volume and the tissue homogenised using a Silverson heavy duty laboratory mixer emulsifier, care being taken to hold all samples on ice. After centrifugation at 800g at 4 °C, the supernatant fraction was removed and stored at -70 °C prior to assay.

2.4.4. Enumeration Of Mast Cell Numbers In Toluidine Blue And rabαSMCP Stained Sequential Sections

Tissue sections were examined using a Leitz Wetzler Dialux 20EB microscope at x500 magnification. For non-mucosal tissues, graticule counts were started from a known point of reference so that the same relative area could be counted and compared on both the toluidine blue and anti-SMCP stained sequential sections. In mucosal tissues cells were counted similarly, cell counts within the lamina propria being spread between the basement membrane and epithelium. A minimum of 50 successive graticule fields were counted on each of two adjacent sequential sections stained with toluidine blue or with anti-SMCP. Four pairs of sections were examined for each tissue. The results were expressed as the mean number of positive mast cells (for toluidine blue or SMCP) per mm^2 of tissue.

2.5. Cutaneous Response Studies

2.5.1. Secretagogue Studies - Agents And Diluents

2.5.1.1. Substance P, Compound 48/80 And Calcium Ionophore A23187

Substance P (sP) was obtained from Cambridge Research Biochemicals Limited, Northwich, UK, Cat.No. B1323 and compound 48/80 (48/80) from Sigma Chemical Co. Ltd., Poole, UK, Cat.No. C4257. Dilutions were made in sterile pyrogen-free isotonic saline (Baxter Healthcare Limited, Thetford, UK) which also served as the diluent control. Dilutions of 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M sP and 1, 10, 25, 50, 100, 250 and 500µg 48/80/ml were used for intradermal injection. Calcium ionophore A23187 (A23187) was obtained from Sigma Chemical Co. Ltd., Poole, UK, Cat.No. C7522. This was initially dissolved in ethanol, final dilutions being made in sterile pyrogen-free isotonic normal saline (Baxter Healthcare Limited, Thetford, UK) as above. The diluent control consisted of saline with the same

proportion of ethanol as present in the highest concentration of A23187 used (10^{-4} M). Dilutions of 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M A23187 were used for intradermal injection.

2.5.1.2. Sheep Mast Cell Proteinase (SMCP)

SMCP was kindly purified by Dr.A.Pemberton, Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Clinical Studies, The University of Edinburgh from the abomasal mucosa of *Telodorsagia circumcincta* infected sheep. Briefly, abomasal samples were collected at post-mortem and stored at -70°C until use. Abomasum (4g) was thawed, minced and homogenised briefly in 20mM Tris/HCl, pH 7.5. The suspension was centrifuged (6000g for 15 minutes at 4°C), the supernatant discarded and the pellet homogenised with 0.667 volumes of 20mM Tris/HCl; 1M NaCl; 0.1% Brij 35 (30% $^{w}/_{v}$ solution, Sigma Chemical Co. Ltd., Poole, UK, Cat.No. 430AG-6); pH 7.5. After centrifugation (6000g for 30 minutes at 4°C) the supernatant was further clarified by centrifugation for 1 hour at 20000g at 4°C. The resulting supernatant was diluted with 2 volumes of 20mM Tris/HCl; 0.1% Brij 35; pH 7.5 and loaded onto a column packed with CM-sepharose FF (25ml) in the same buffer. The column was then eluted with a 600mM NaCl gradient and the fractions assayed for chymotrypsin-like activity by adding 10µl of each fraction to 50µl of 100mM Tris/HCl pH 7.5 in a 96 well ELISA plate, followed by 10µl of 10mM N-succinyl-ala-ala-pro-phe-*p*-nitroanilide (Sigma Chemical Co. Ltd., Poole, UK, Cat.No. S 7388) in dimethyl sulphoxide. Active fractions appear yellow after a short incubation at room temperature. The active fractions were pooled, diluted with 2 volumes of 20mM Tris/HCl; 0.1% Brij; pH 7.5 and loaded onto a Mono S cation exchange column in the same buffer. This column was eluted in a 150-350 mM NaCl gradient, and the active fractions pooled for further chromatography on Mono S in 20mM phosphate buffer; 0.1% Brij 35; pH 7.0. SMCP was eluted in a 50-400mM NaCl gradient, the SMCP diluent control being composed of the same proportions of 20mM phosphate buffer; 0.1% Brij 35; pH 7.0 and NaCl concentration as at

the point of SMCP elution. A doublet (representing SMCP) was detectable on a coomassie blue-stained SDS-PAGE gel (Fig.2.3). (This was generously undertaken by Dr. Alan Pemberton, RDSVS, utilising discontinuous SDS-PAGE (Laemmli 1970) on a 12% gel that was electrophoresed for 3hr at 5°C at a constant current of 20mA per gel). 36µg, 3.6µg, 360ng and 36ng SMCP were used for intradermal injection. SMCP and SMCP diluent control solutions were negative for the presence of endotoxin (kindly undertaken by Dr.Gordon Moon of the Moredun Research Institute, Edinburgh).

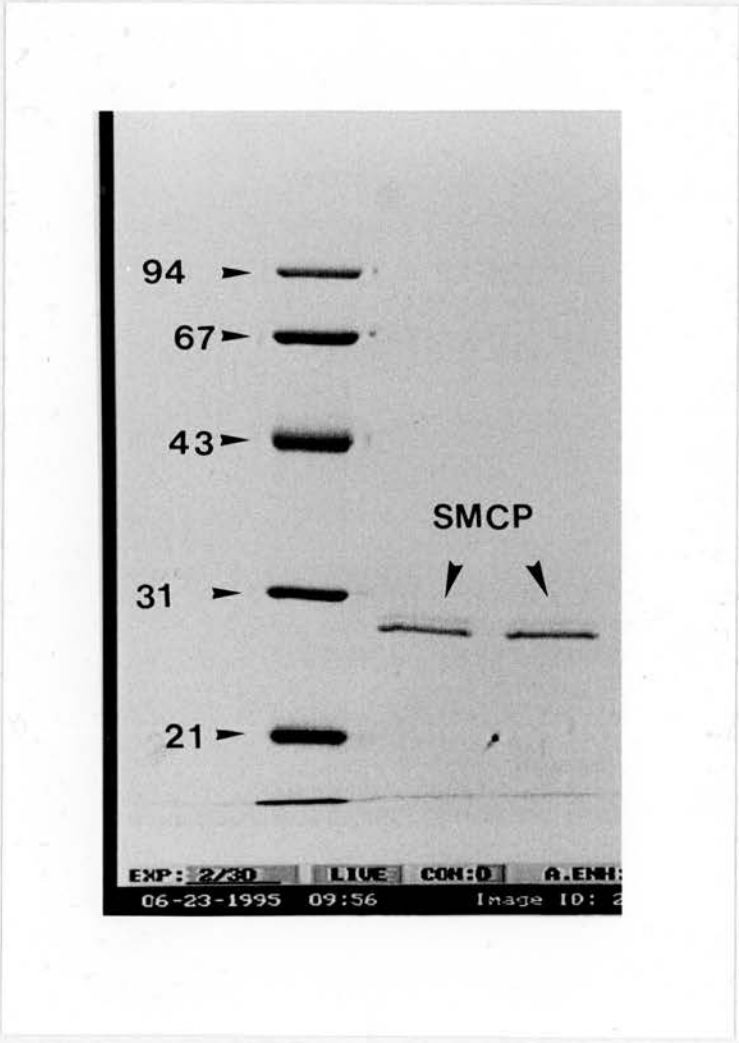


Figure 2.3. SDS-PAGE gel (12%) of purified SMCP from ovine abomasal tissue stained with coomassie brilliant blue R250. Note the doublet at approximately 25kDa, representing SMCP. This indicates a glycoform of SMCP, which is also apparent when the monoclonal antibody to SMCP is used. Arrows indicate the positions of the protein standards at 94kDa, 67kDa, 43kDa, 31kDa and 21kDa.

2.5.1.3. Preparation Of Heat-Inactivated SMCP (HI-SMCP) And Heat-Inactivated Diluent Control

Aliquots of SMCP and SMCP diluent control were placed in a water bath at 64°C for 10 minutes. Chymotryptic activity of active and heat-inactivated SMCP was assessed by cleavage of N-succinyl-ala-ala-pro-phe-*p*-nitroanilide in dimethyl sulphoxide to *p*-nitroaniline, the optical density being read at 405nm, duplicate samples of both agents being used. Therefore, 10µl of sample was added to 20µl of 10mM N-succinyl-ala-ala-pro-phe-*p*-nitroanilide in dimethyl sulphoxide and 170µl of 20mM phosphate buffer; 0.1% Brij 35; pH 7.0. The rate of reaction for each sample was determined in a Beckman Spectrophotometer at 405nm. The percentage residual activity in heat-inactivated SMCP was calculated as follows:-

Percentage Residual Chymotryptic Activity (%) =

$$\frac{[(\text{Cleavage rate inactive SMCP sample 1} + \text{Cleavage rate inactive SMCP sample 2})]/2}{[(\text{Cleavage rate active SMCP sample 1} + \text{Cleavage rate active SMCP sample 2})]/2} \times 100$$

Representative results (26/1/95 and 23/6/95) for SMCP after heat-inactivation gave residual activities of 2.4% and 1.35% respectively.

All of the above agents and diluents were stored at -70 °C as stock solutions, and were thawed and made up to working dilutions immediately prior to use. All dilutions were kept on ice until shortly prior to injection.

2.5.2. Intradermal Skin Testing

2.5.2.1. Method

An area of skin on the left or right flank was shaved on the day prior to undertaking the study. For ethical reasons, the number of injection sites per flank was restricted to six, these being marked in a three by two grid with a felt-tip pen (Figures 2.4. and 2.5.). (It should be noted that benzalkonium chloride, which can be used in routine skin preparation and cleansing (Debuf 1994), is a competitive antagonist of 48/80 (Read et al 1982) and was therefore

avoided for use in the studies described in this thesis). 50µl of the appropriate agent or diluent control was injected intradermally at each site using a 27 gauge 1ml syringe (Myjector®; Terumo, Leuven, Belgium, Cat.No. SS-NIH273) and monitored for weal development.

2.5.2.2. Measurement Of Weal Volume

Using an adapted low-tension spring-loaded micrometer (Draper®, UK, Cat.No. SI-510) the skin thickness was measured at the injection site before and at time points after injection of the agent or diluent. The diameter of the test weal response at the appropriate time point was measured in two horizontal planes at right angles to each other and a mean value of weal diameter calculated. The weal volume was determined from the weal thickness and weal area (Coulson and Holden 1990; formulae below) and expressed in mm^3.

$$\text{Weal Area} = \pi/_4[(d_1 + d_2)/2]^2$$

$$\text{Weal Thickness} = (T_1 - T_0)/2$$

$$\text{Weal Volume} = \text{Area} \times \text{Thickness}$$

(Where d_1 is the maximum horizontal weal diameter and d_2 the weal diameter at right angles to it. T_0 is the skin thickness prior to the intradermal injection of the agent or diluent. T_1 is the skin thickness measured at the appropriate time after the intradermal injection of the agent or diluent).

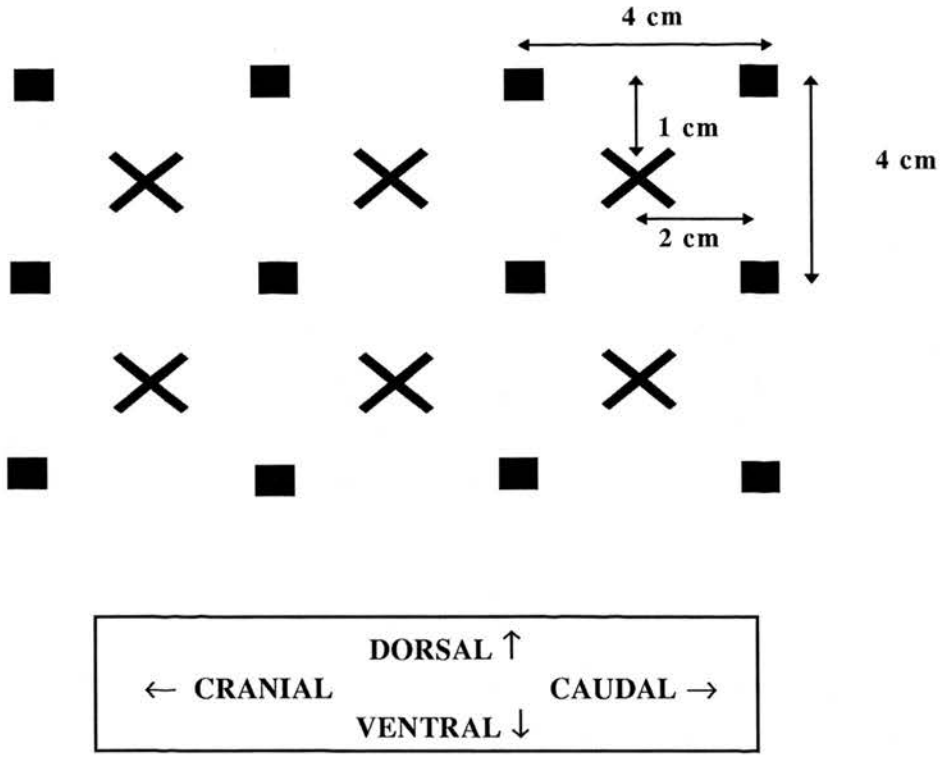


Figure 2.4. Diagram of intradermal injection sites. Injection sites are marked by '**X**'.

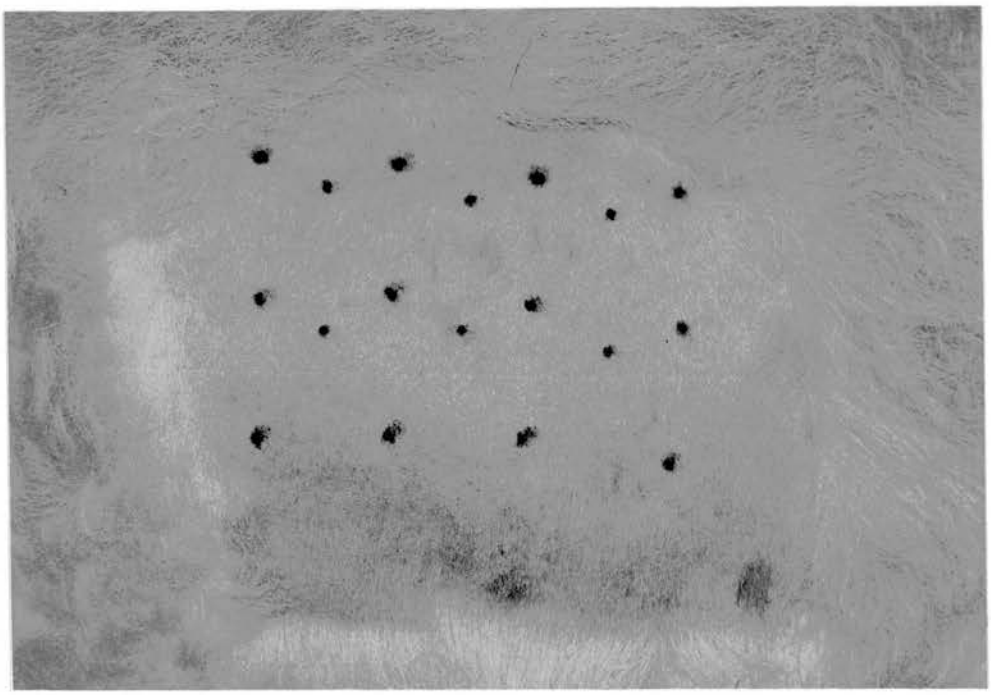


Figure 2.5. Shaved left flank of ewe with grid marked (as outlined above) for intradermal skin testing.

2.5.2.3. Skin Biopsy

Local anaesthetic (Lidocaine hydrochloride, 1ml Xylocaine 2%® per injection site; Astra Pharmaceuticals, Kings Langley, UK) without adrenaline (Henfrey, Thoday and Head 1991) was injected subcutaneously prior to biopsy collection. Biopsies were excised using sterile, disposable 6mm skin biopsy punches (Kruuse, The Netherlands). Each biopsy site was closed using a single Michel surgical clip (12 mm Martin 100 Michel clips, Germany). In all cases, healing was unremarkable and complete by 10 days post-biopsy.

2.5.3. Cell Counts On Skin Sections

A Leitz Wetzler Dialux 20 EB microscope was used at a magnification of 500x to enumerate mast cell (toluidine blue stained sections), neutrophil (haematoxylin and eosin stained sections) and eosinophil (carbol chromatrope stained sections) numbers. Moving from epidermis to dermis in a consistent grid pattern to a maximum depth of 10 graticule field widths (generally the limit of the deep dermis)[Figure 2.6.], the appropriate cells in 20 graticule fields (equivalent to a total area of 1.51 mm^2 per section) were counted for each section and expressed as cells mm^{-2} skin. The grid pattern ensured that the same graticule area of skin was not counted twice, and that all areas of epidermis, superficial and deep dermis would be included in cell counts. This pattern also reduced the possibility of operator bias by not allowing the preferential inclusion of foci of cellular infiltrates in counts. The observer was blinded to the identity of individual sections until after the counts for a particular experiment had been completed. This ensured that the operator was blinded to the experimental protocol. On occasion where the skin thickness was less than ten graticule field widths, the same grid counting pattern was followed, but extended upon reaching the epidermis.

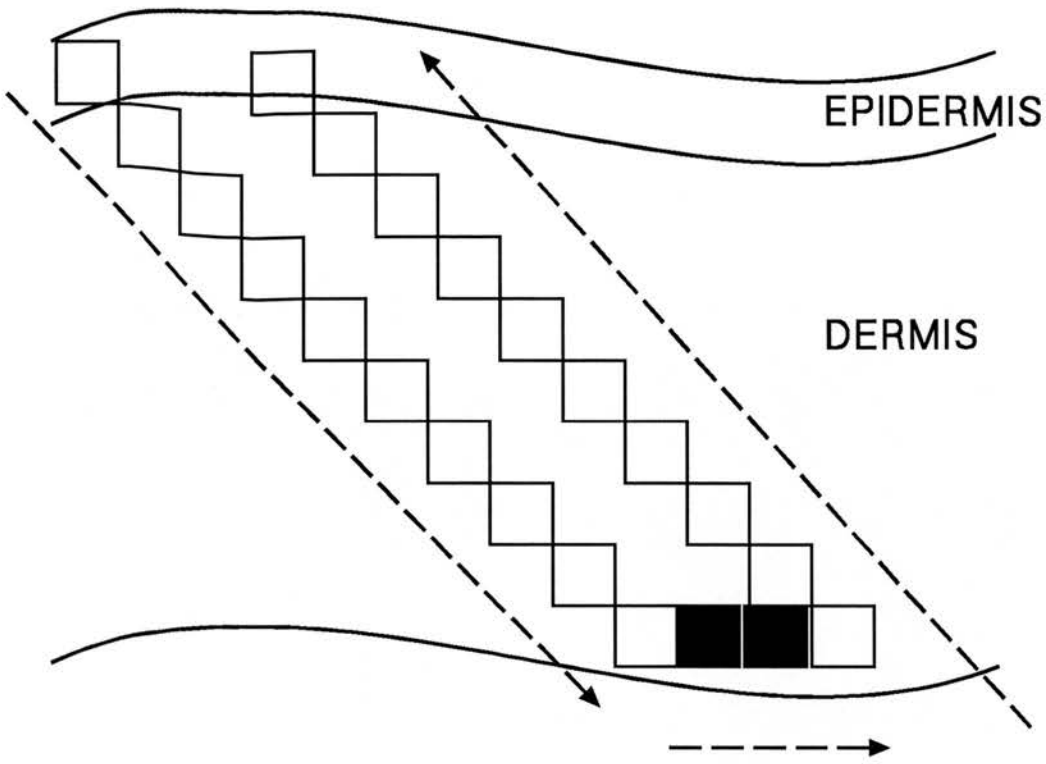


Figure 2.6. Diagram depicting consistent graticule grid counting pattern for enumeration of cell numbers in skin sections (20 graticule fields equivalent to 1.51mm^2 were counted for each section, represented by the open squares above).

2.5.4. Subjective Assessment Of The Extent Of Mast Cell Degranulation In Toluidine Blue Stained Tissue Sections

In order to assess the extent of mast cell degranulation, between 50 and 150 toluidine blue stained mast cells (dependant on the section) were examined with a Leitz Wetzler Dialux 20 EB microscope at x1000 magnification. Cells were allocated to one of three separate categories :- 1) non-degranulated cells (up to 10% degranulation), 2) moderately degranulated cells (10% to 50% degranulation) and 3) extensively degranulated cells (greater than 50% degranulation) (adapted from Iwamoto et al [1992]). The percentage of extensively degranulated cells per tissue section was subsequently calculated.

2.5.5. Computer Hardware/Software

The thesis was compiled on a Dell® Dimension 450Si computer (Dell Computers Ltd, Eire). Text was written on Microsoft® Word 6.0 for Windows™, data being analysed using Microsoft® Excel 5.0 for Windows™, Fig.P® 1.0 for Windows™ (Biosoft®, Cambridge, UK), and Minitab® version 7.2. The Reference Manager® 5.0 program (Research Information Systems Inc., USA), in conjunction with the on-line BIDS database (accessed by e-mail), was used for reference handling.

2.5.6. Photography

Photomicrographs were taken on a Leitz Wetzler Ortholux II microscope system, using Ilford FP4 Plus 125 ASA black and white and Kodak Ektachrome 64T EPY (Professional) colour film. Photographs were taken predominantly using a Canon EOS 1000FN SLR camera and Ektachrome film.

2.5.7. Statistical Analyses

The sample mean, standard deviation (SD) and standard error of the mean (SEM) were calculated on a standard scientific calculator.

Data analysis was undertaken using the Minitab® statistical program version 7.2 (Minitab Inc., State College, PA, USA). In order to determine whether the data within samples was normally distributed, the data was tested for normality of distribution using a method approximating to the Shapiro-Wilk test. This calculates the degree of correlation between the actual distribution of data and a theoretically calculated normal distribution of the same data. By reference to a table of probability values (Minitab® Reference Manual) the likelihood of the data approximating a normal distribution can be ascertained. Dependant on the result, the data was subsequently analysed using the following tests.

2.5.7.1. Student's *t* - Test

Where data within samples approximated to a normal distribution, the Student's two sample *t* test was performed using the Minitab® program.

2.5.7.2. Mann-Whitney U Test

Where data within did not approximate to a normal distribution, the non-parametric Mann-Whitney U test was applied, using the Minitab® program.

In the above tests, $P \leq 0.05$ was taken to denote statistical significance.

2.5.7.3. Linear Regression Analysis

This was performed using Fig.P® software (Biosoft®, Cambridge, UK).

2.5.7.4. Analysis Of Variance (ANOVA)

The analysis of variance between groups of data was undertaken using the Minitab® program.

CHAPTER 3

THE TISSUE DISTRIBUTION OF SHEEP MAST CELL PROTEINASE : THE ELUCIDATION OF OVINE MAST CELL HETEROGENEITY

3.1. Introduction

3.1.1. Chymases and mast cell heterogeneity - general points

Mast cells in rodents and man represent a phenotypically heterogeneous cell population with respect to tissue site, fixation properties, histochemical staining, biochemistry and functional activity (Galli 1990). These differences are a reflection of differences in their granule constituents, most notably in their content of intra-granule proteinases. Chymotrypsin-like mast cell proteinases (chymases) have been isolated from mast cells of the rat (Lagunoff and Pritzl 1976, Yurt and Austen 1977, Woodbury et al 1978), mouse (Newlands et al 1987, Reynolds et al 1990), dog (Slavin et al 1987, Caughey et al 1988b, Schechter et al 1988), sheep (Huntley et al 1986) and man (Schechter et al 1983, Schechter et al 1986). The elucidation of differences in mast cell proteinase expression at different tissue sites has led to the identification of mast cell subsets, as outlined below, in the rat (Gibson & Miller 1986, Gibson et al 1987, Huntley et al 1990), mouse (Miller et al 1988, Miller et al 1989, Reynolds et al 1990) and man (Irani et al 1986, Schwartz et al 1987, Schwartz 1989).

3.1.2. Rat chymases

In the rat and mouse, distinct proteinases are predominantly associated with either the mucosal mast cell (MMC) or connective tissue mast cell (CTMC) subsets. Rat mast cell proteinase-II (RMCP-II) is chiefly associated with the gastrointestinal MMC subset (Woodbury, Gruzenski and Lagunoff 1978), whereas rat mast cell proteinase-I (RMCP-I) is predominantly associated with the CTMC subset. Thus, the mucosa of the stomach and small intestine contains exclusively RMCP-II staining mast cells, whereas the submucosa at these sites contains 63% and 100% of RMCP-I staining cells respectively (Huntley et al 1990). Uniquely RMCP-I containing CTMC-type mast cell populations have been defined in tongue and in small intestinal submucosa (Huntley et al 1990). The presence of RMCP-II in non-mucosal sites such as liver and thymus (Gibson and Miller 1986), and the identification of dual RMCP-I

and RMCP-II staining cells in gastric submucosa, liver, mesenteric lymph node, peribronchiolar region of the lung and pleura also indicates that proteinase heterogeneity exists *within* rat mast cell subpopulations (Huntley et al 1990).

3.1.3. Mouse chymases

In the mouse, five chymases have currently been identified (Reynolds et al 1990), mouse mast cell proteinase-1 (MMCP-1) and mouse mast cell proteinase-2 (MMCP-2) being present in the MMC subset, whereas MMCP-3, MMCP-4 and MMCP-5 delineate the connective tissue subset (Newlands et al 1993, Stevens et al 1994, Schwartz 1994). However, it is known that proteinase heterogeneity exists within both the intestinal (MMC; Miller et al 1988) and cutaneous (CTMC; Stevens et al 1994) mouse mast cell subpopulations. For example, MMCP-2 (as well as MMCP-5) can be detected in murine cutaneous mast cells (Stevens et al 1994).

3.1.4. Human chymase

Mast cell subpopulations in man are divided into those that contain tryptase and chymase (the MC^{TC} type) and those that contain tryptase alone (the MC^{T} type) (Irani et al 1986). Most tissues are believed to contain a mixture of both MC^{TC} and MC^{T} types, although MC^{T} is predominant in the lung and small intestinal mucosa and MC^{TC} is chiefly found in the skin and small intestinal submucosa (Irani et al 1986, Schwartz et al 1987, Schwartz 1989, Irani and Schwartz 1990). However, the validity of the belief that human mast cell heterogeneity can be based upon differences in proteinase composition has recently been challenged (Aldenborg and Enerbäck 1994).

3.1.5. Canine chymase

Although it is known that canine cutaneous mast cells contain both a chymase and a tryptase (Schechter et al 1988), little is known regarding mast cell heterogeneity at other tissue sites in this species.

3.1.6. Ovine chymase

Sheep mast cell proteinase (SMCP) has been isolated and characterised as a serine endopeptidase (Knox and Huntley 1987), and the mast cell source of this enzyme confirmed immunohistochemically (Huntley et al 1986). SMCP is released locally into lymph and systemically into blood following abomasal challenge with gastric nematodes in immune sheep (Huntley et al 1987). Indeed, although the concentration of SMCP in the gastrointestinal mucosa is significantly correlated with mast cell counts (Huntley 1991), the distribution of SMCP in non-mucosal tissues was unknown. Additionally, it was not known whether mast cell heterogeneity existed in the sheep.

3.2. Experiment 3.1 - Investigation Of The Tissue Distribution Of SMCP In The Sheep

3.2.1. Experimental Aim and Design

The aims of the experiment were therefore to determine the tissue distribution of SMCP in both mucosal and non-mucosal sites, and to establish whether or not mast cell heterogeneity, as a consequence of differences in the distribution of SMCP between tissues, was present. These aims were accomplished by firstly determining whether or not SMCP was present in tissues by immunohistochemistry and ELISA (using an affinity purified, polyclonal RabαSMCP antibody), and secondly by comparing the number of mast cells identified histochemically (toluidine blue pH 0.5) with the number detected immunohistochemically on sequential tissue sections.

At post-mortem examination, samples from trachea, bronchial lymph node, lung, thymus, spleen, liver, flank skin, abomasum, duodenum, jejunum, ileum, colon and mesenteric lymph node were removed from four Suffolk-cross lambs. Samples were weighed, homogenized in buffer, and the amount of SMCP present determined by ELISA (2.4.3, 2.3.12.1). This was expressed as ng SMCP per g wet weight of tissue. Pairs of sequential sections were stained histochemically with toluidine blue pH 0.5 and immunohistochemically to demonstrate the presence of SMCP (2.4.2), using either the 3,3'-Diaminobenzidine (DAB) method or, in heavily pigmented skin, the Vectastain® ABC-AP method. The use of the latter technique allowed the distinction between immunostaining of SMCP in dermal mast cells and endogenous melanin in dermal melanocytes to be made. A minimum of 50 successive graticule fields covering the same area on both the toluidine blue and anti-SMCP stained sequential sections were counted (2.4.4), the resultant counts being compared for each tissue using the Mann-Whitney U test and for all tissues using linear regression analysis. Additionally, the number of SMCP-positive mast cells were compared against the respective tissue SMCP concentrations, by the use of linear regression analysis.

3.2.2. Results

The median and ranges for the toluidine blue and SMCP mast cell counts, and for the SMCP mast cell counts and respective tissue SMCP concentrations, are given in Tables 3.1 and 3.2 respectively. The highest median mast cell counts (215.8/mm^2 with toluidine blue and 211.2/mm^2 with anti-SMCP), and the highest median concentration of SMCP (24.76µg SMCP/g wet tissue) were in the thymus which contained diffusely scattered focal accumulations of cells, with a predominantly peri-vascular distribution (Fig. 3.1). The gastrointestinal tissues as a group had relatively high cell counts with both techniques (Fig. 3.2), and comparatively high tissue SMCP concentrations (Table 3.2). Despite appreciable numbers of mast cells determined by both histochemical (Fig. 3.3) and

immunohistochemical means, SMCP was not detected in tracheal homogenates by ELISA (Table 3.2). The cells in this instance were chiefly subepithelial in location.

		Number of toluidine blue-positive mast cells per mm^2 of tissue		Number of SMCP-positive mast cells per mm^2 of tissue	
Tissue Examined	**n**	**Median**	**Range**	**Median**	**Range**
Gastrointestinal System					
Abomasum	4	**41.4**	21.0-223.7	**43.3**	24.9-250.5
Duodenum	3	**52.9**	20.1-168.5	**78.4**	25.3-146.0
Jejunum	4	**188.3**	90.2-205.8	**173.2**	78.4-194.8
Ileum	4	**109.1**	43.6-242.0	**118.2**	34.8-241.4
Colon	4	**37.0**	35.2-152.9	**35.3**	27.5-124.8
Respiratory System					
Trachea	3	**19.6**	13.8-25.9	**24.9**	13.2-28.6
Bronchus	3	**24.3**	18.0-31.2	**23.8**	16.4-31.2
Bronchial Lymph Node	4	**48.9**	20.9-128.5	**55.6**	19.6-111.4
Lung	4	**39.7**	20.6-100.0	**39.7**	22.2-103.7
Other Tissues					
Thymus	3	**215.8**	190.9-270.3	**211.2**	210.6-221.6
Spleen	4	**55.5**	48.8-117.1	**46.6**	42.1-84.6
***Liver* ***	*4*	***89.1***	*46.0-98.9*	***14.3***	*8.89-15.2*
***Skin* ***	*4*	***43.3***	*26.6-55.3*	***0.7***	*0.0-2.1*
Mesenteric Lymph Node	4	**131.7**	64.0-225.2	**121.6**	49.8-244.5

Table 3.1. Table comparing the median and range values for the number of toluidine blue and SMCP-positive mast cells per mm^2 tissue for all tissues examined. (* represents statistical significance for the number of toluidine blue- versus SMCP-stained mast cells at $P<0.05$ assessed by the Mann-Whitney U test).

		Number of SMCP-positive mast cells per mm^2 of tissue		Tissue SMCP concentration (µg SMCP per g of wet tissue)	
Tissue Examined	**n**	**Median**	**Range**	**Median**	**Range**
Gastrointestinal System					
Abomasum	4	**43.3**	24.9-250.5	**0.08**	0.00-0.32
Duodenum	3	**78.4**	25.3-146.0	**1.40**	0.00-2.42
Jejunum	4	**173.2**	78.4-194.8	**0.89**	0.69-2.31
Ileum	4	**118.2**	34.8-241.4	**2.29**	0.85-7.92
Colon	4	**35.3**	27.5-124.8	**0.60**	0.09-0.98
Respiratory System					
Trachea	3	**24.9**	13.2-28.6	**0.00**	0.00-0.00
Bronchus	3	**23.8**	16.4-31.2	**0.00**	0.00-0.15
Bronchial Lymph Node	4	**55.6**	19.6-111.4	**0.10**	0.03-0.25
Lung	4	**39.7**	22.2-103.7	**0.09**	0.07-0.18
Other Tissues					
Thymus	3	**211.2**	210.6-221.6	**24.76**	1.66-32.81
Spleen	4	**46.6**	42.1-84.6	**0.07**	0.03-0.14
Liver	4	**14.3**	8.89-15.2	**0.00**	0.00-0.02
Skin	4	**0.7**	0.0-2.1	**0.01**	0.00-0.06
Mesenteric Lymph Node	4	**121.6**	49.8-244.5	**0.37**	0.04-0.45

Table 3.2. Table comparing the median and range values for the number of SMCP positive mast cells per mm^2 against the tissue SMCP concentration (µg SMCP per g wet tissue) for all tissues examined.

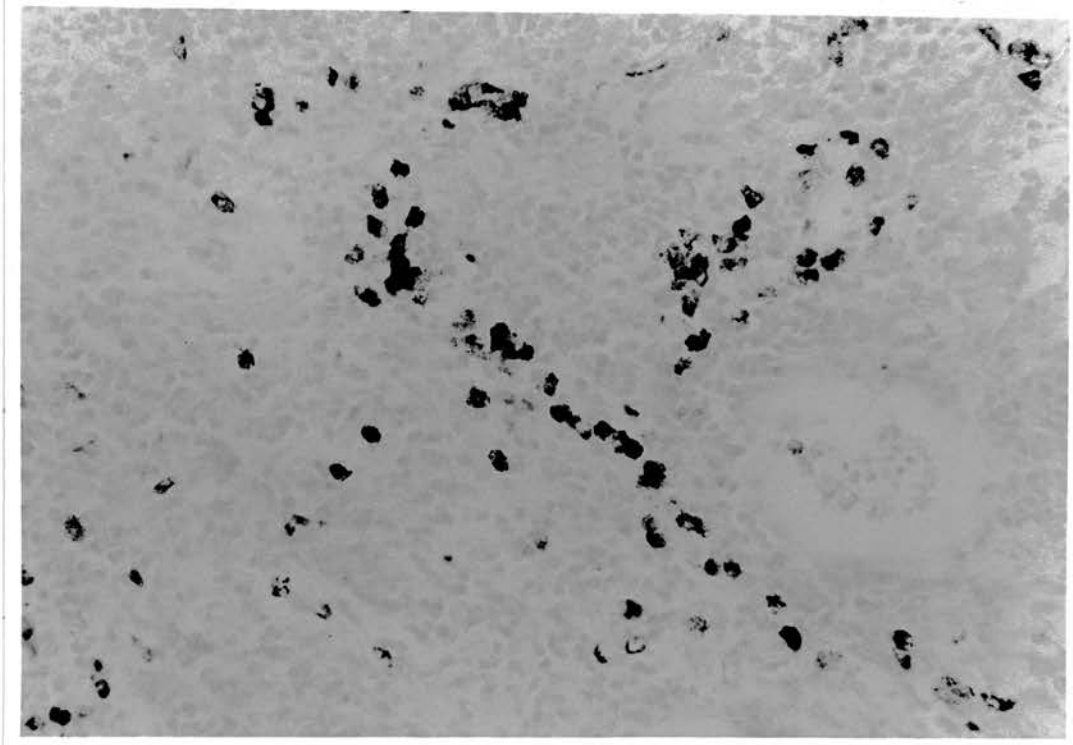


Figure 3.1. Toluidine blue-stained section of ovine thymus (x250). Note large numbers of darkly stained mast cells focally distributed throughout the section.

Figure 3.2. Toluidine blue-stained section of ovine jejunum (x400). Numerous mast cells are present.

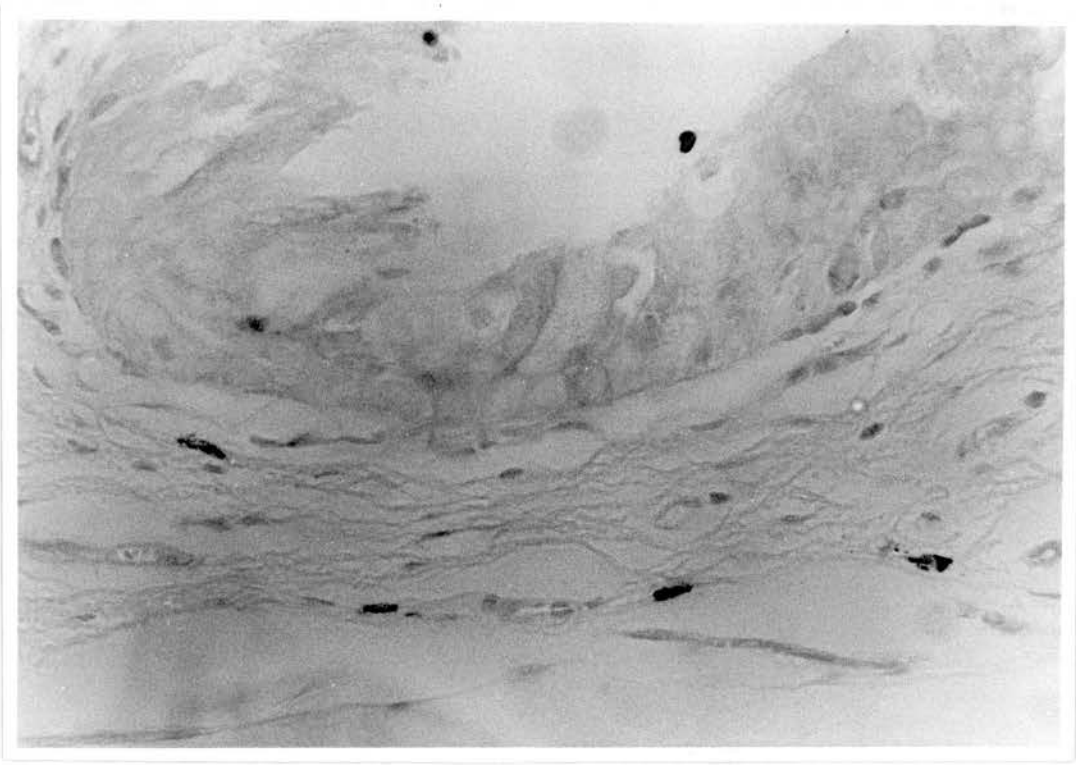


Figure 3.3. Toluidine blue-stained section of ovine trachea (x400). Note predominantly subepithelial location of mast cells.

There were fewer SMCP-positive cells in comparison to toluidine blue-positive cells in both skin ($p<0.05$) and liver ($p<0.05$) (Table 3.1). No statistically significant differences were observed for the other individual tissues examined. For direct comparison, representative sections of thymus and skin stained with toluidine blue and for SMCP are shown (Fig. 3.4). The anti-SMCP reactive dermal cells were morphologically identical to mast cells (well granulated, mononuclear cells). Dermal basophils were infrequently observed. Their distinct polymorphonuclear appearance and smaller, finer granules allowed them to be differentiated histologically from mast cells. In the liver, far fewer cells (16%) reacted with antibody than with toluidine blue. Morphological examination suggested that many of the toluidine blue stained cells were likely to be large granulated lymphocytes (LGL).

There was a positive correlation between toluidine blue and SMCP mast cell counts ($r^2 = 0.96$, $p<0.001$; Fig. 3.5) and between SMCP cell counts and tissue SMCP concentrations ($r^2 = 0.30$, $p<0.02$; Fig. 3.6) from gastrointestinal tissues.

When the number of cells stained by toluidine blue from all tissues except liver and skin was compared to the number of SMCP-stained cells in the same tissues there was a high degree of correlation ($r^2 = 0.96$, $p<0.001$; Fig. 3.7), in contrast to the finding in skin and liver. A statistically significant correlation was observed between overall SMCP cell counts and concentrations of SMCP in all of the tissues (excluding trachea) [$r^2 = 0.23$, $p<0.001$].

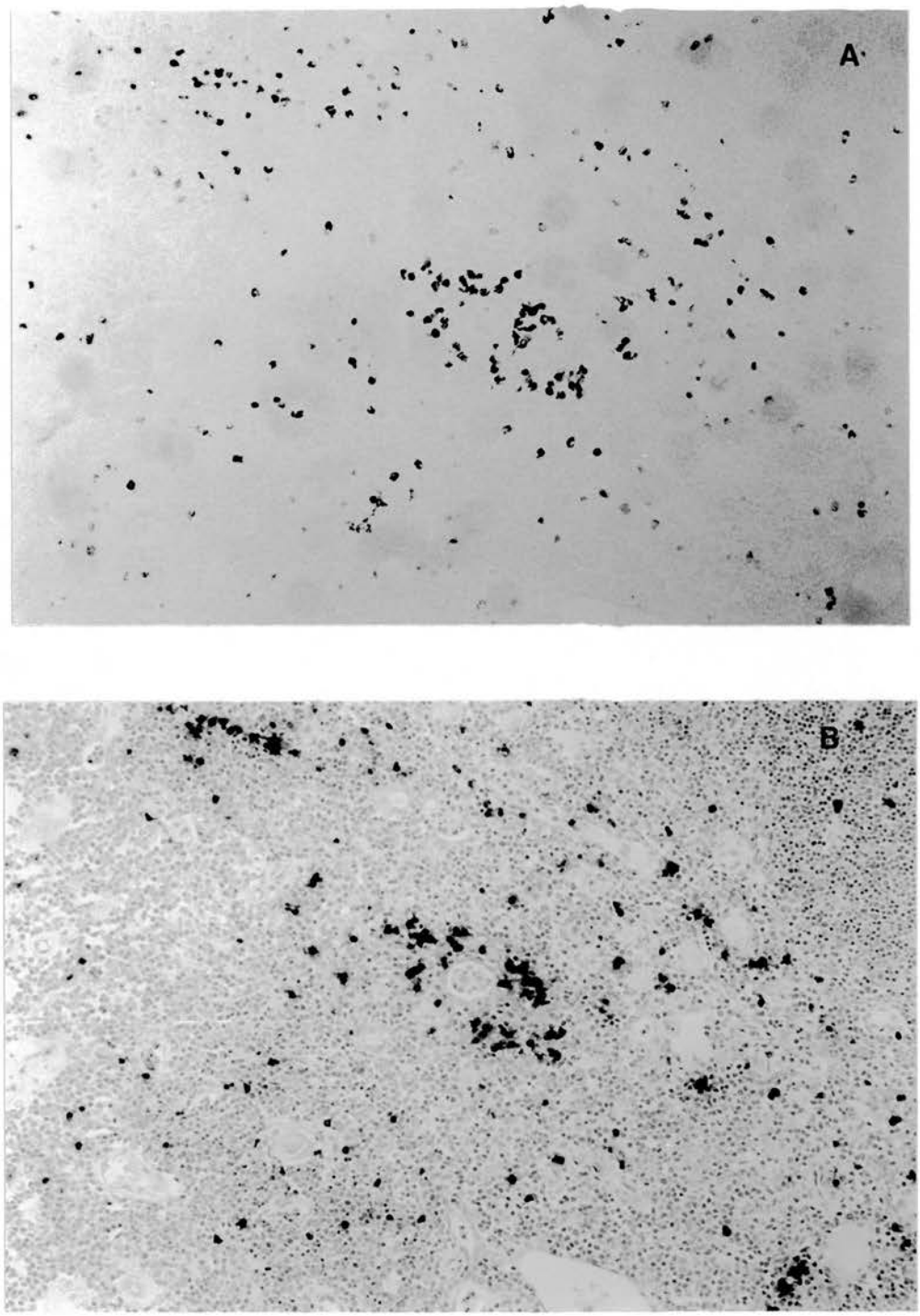


Figure 3.4(a) and (b). Toluidine blue **(a)** and anti-SMCP **(b)** stained sections of ovine thymus (x125). Note that relative numbers of mast cells detectable by both methods is similar.

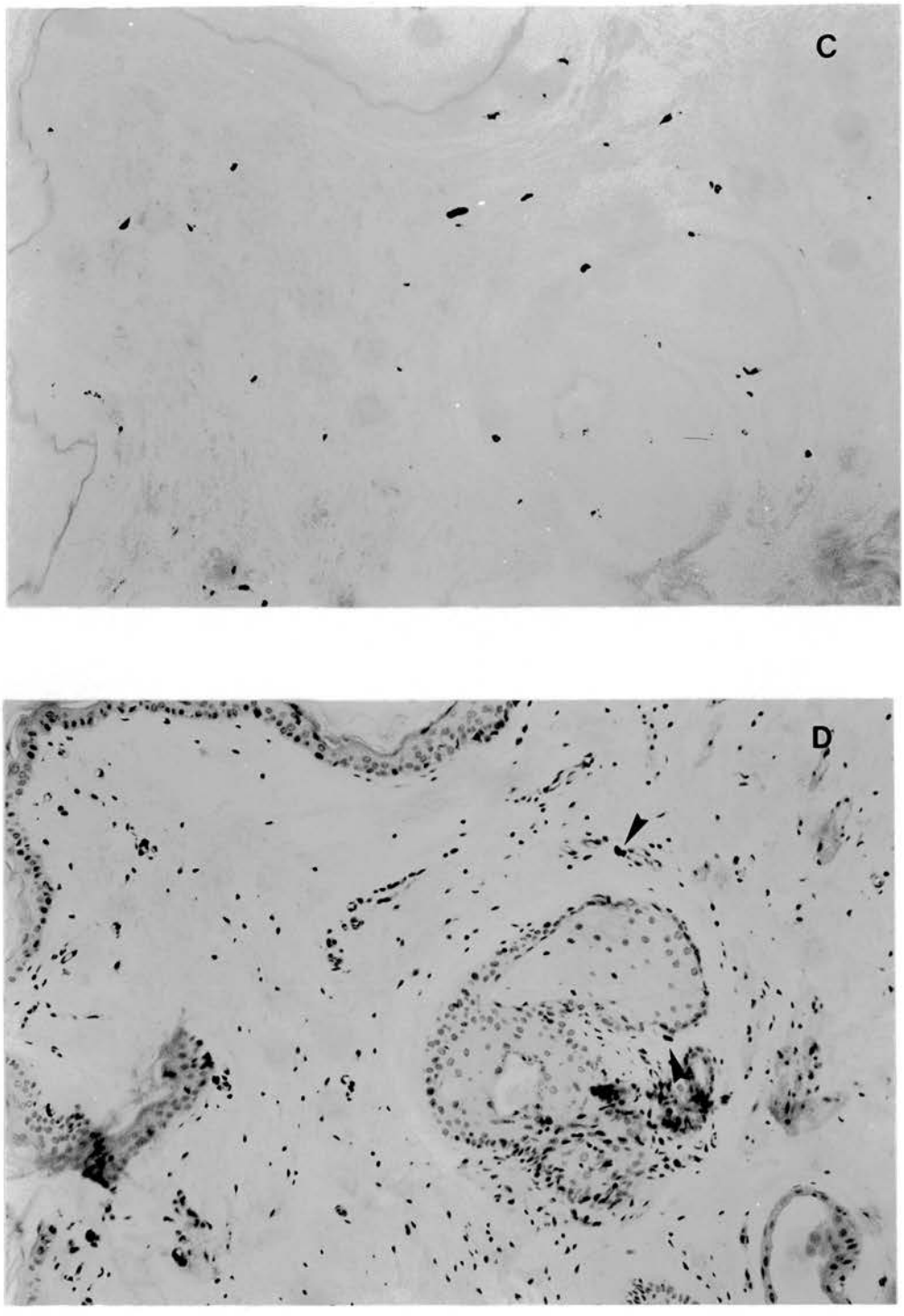


Figure 3.4(c) and (d). Toluidine blue **(c)** and anti-SMCP **(d)** stained sections of ovine skin (x125). Note the disparity in mast cell numbers detectable by the two methods.

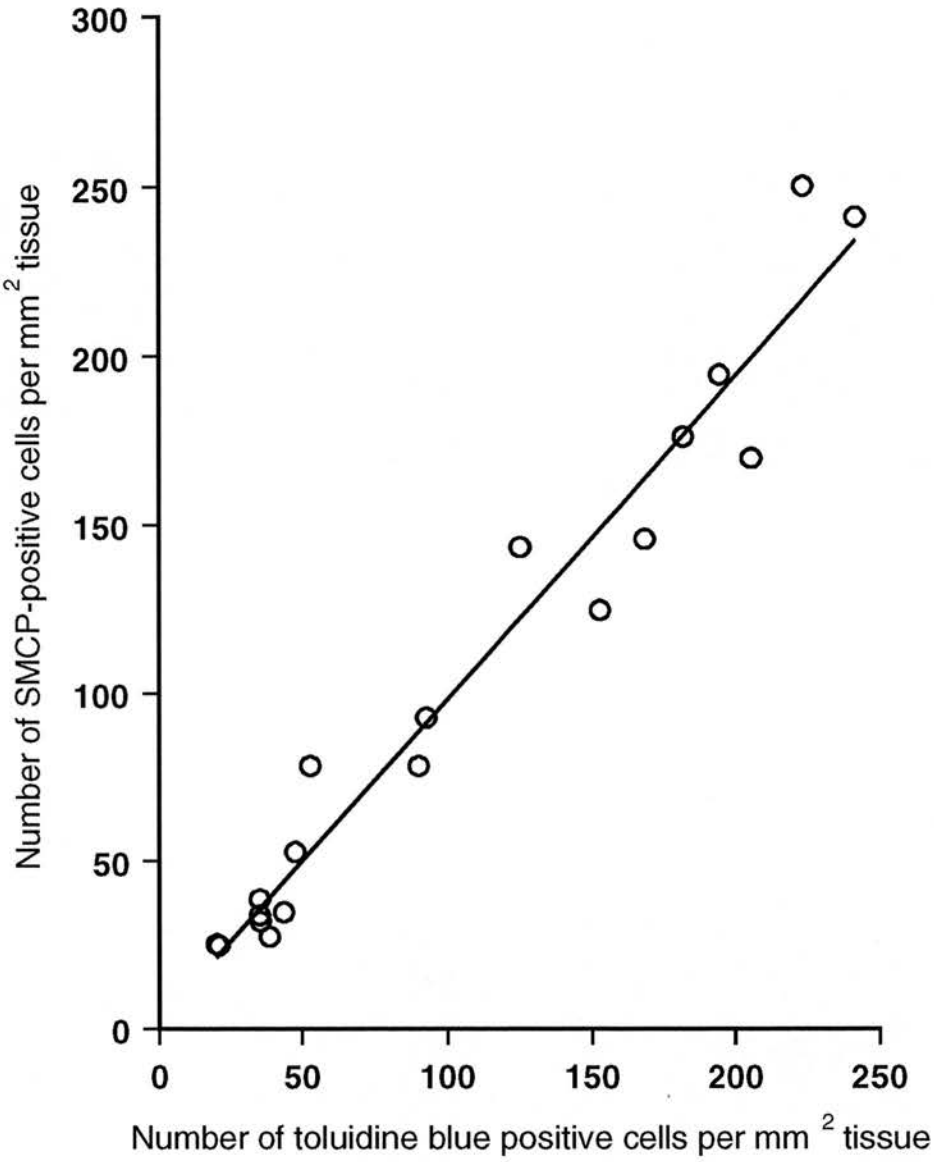


Figure 3.5. Comparison of the number of toluidine blue and SMCP-positive mast cells per mm^2 tissue in the gastrointestinal tract (abomasum, duodenum, jejunum, ileum, colon). Each datapoint represents the counts from sequential sections from an individual tissue. ($r = 0.98$, $r^2 = 0.96$, $p < 0.001$).

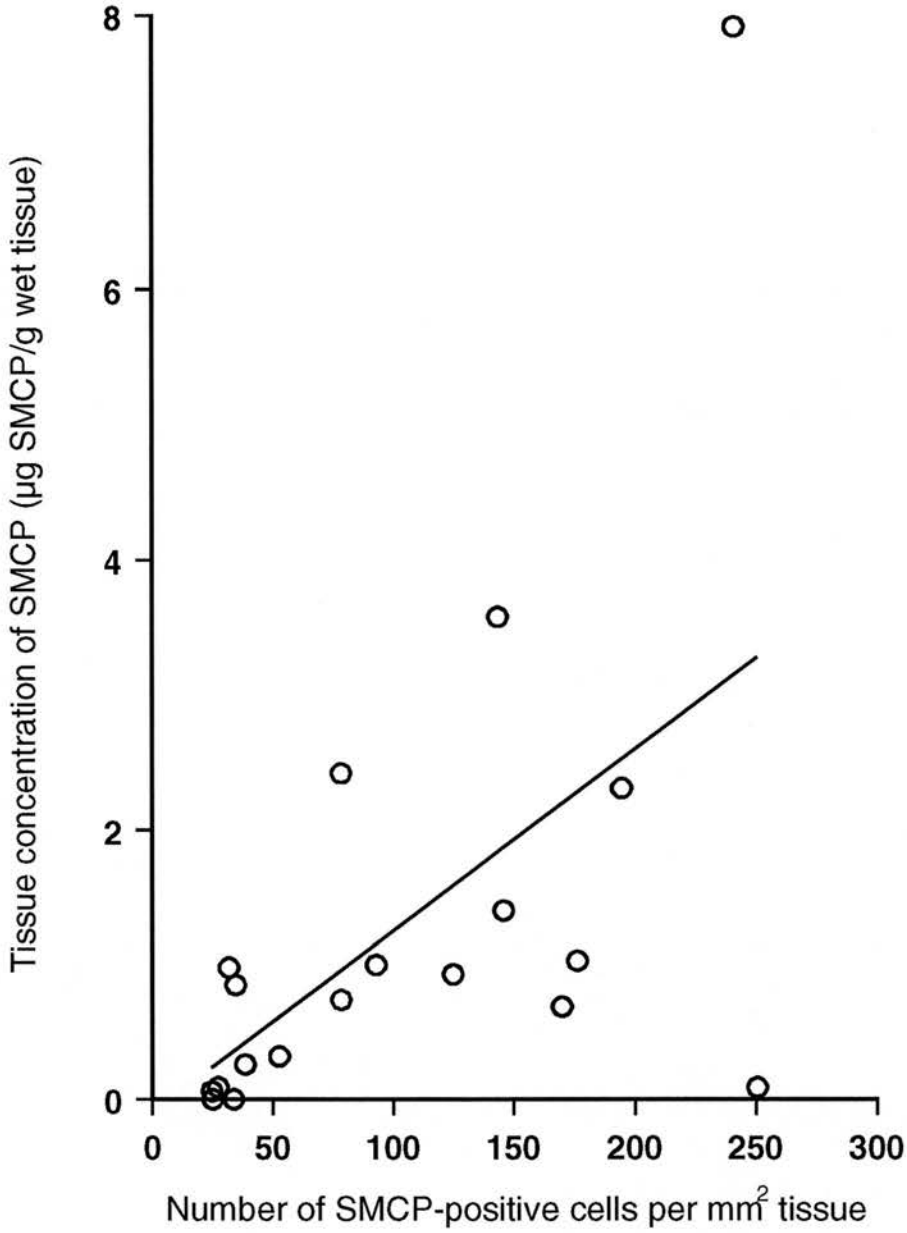


Figure 3.6. Comparison of the number of SMCP-positive cells per mm^2 tissue versus the concentration (µg SMCP/g wet tissue) of SMCP in the gastrointestinal tract (abomasum, duodenum, jejunum, ileum, colon). Each datapoint represents values for an individual tissue ($r = 0.55$, $r^2 = 0.3$, $p < 0.02$).

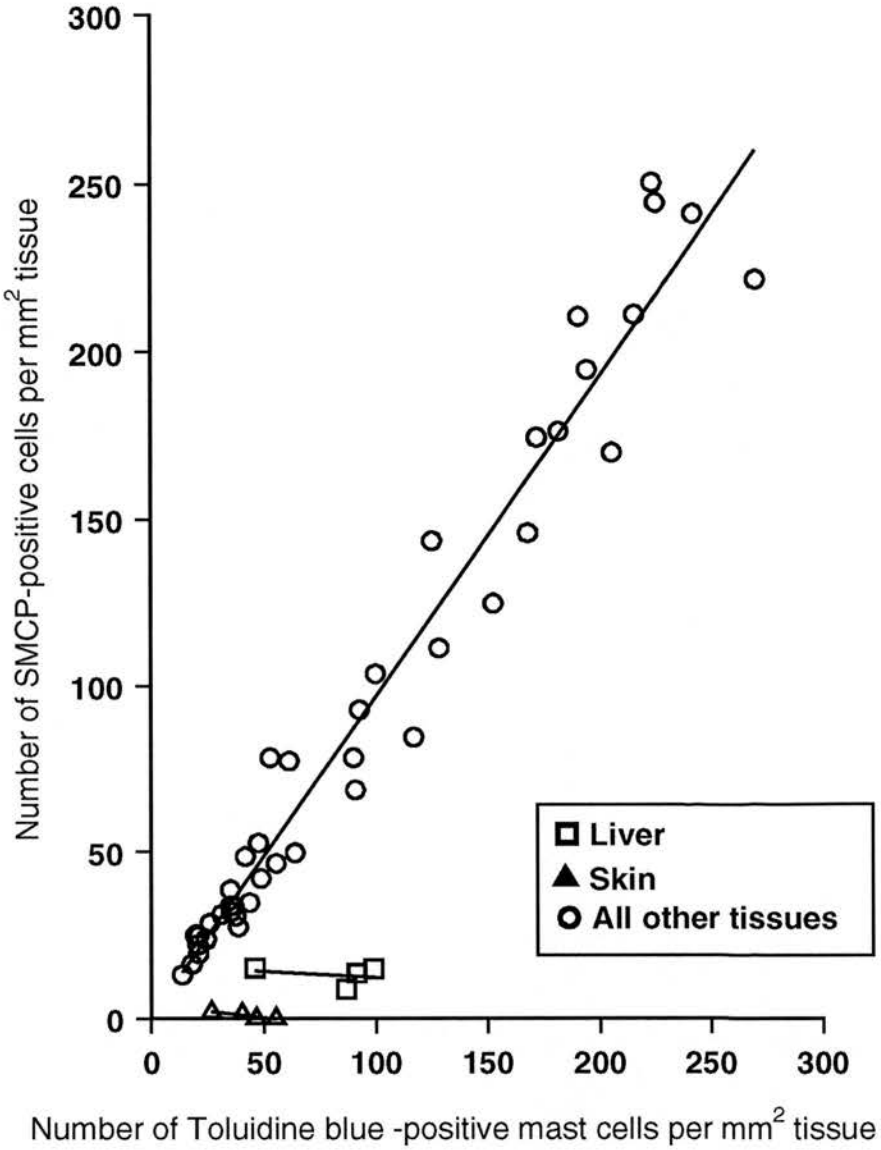


Figure 3.7. Comparison of the numbers of Toluidine blue- and SMCP-positive mast cells per mm^2 for all tissues. The regression line is calculated including all tissues except liver and skin ($r = 0.98$, $r^2 = 0.96$, $p < 0.001$). Each datapoint represents the counts for sequential sections from an individual tissue.

3.3. Discussion

Mast cells were detected with the anti-SMCP antibody in virtually all of the tissues examined, but SMCP positive cells were scarce in skin. This indicates the presence of two distinct mast cell populations in sheep, this finding being in accordance with previous studies demonstrating mast cell proteinase heterogeneity in the rat (Gibson and Miller 1986, Gibson et al 1987, Huntley et al 1990), mouse (Reynolds et al 1990, Newlands et al 1993, Stevens et al 1994) and man (Irani et al 1986, Schwartz et al 1987, Schwartz 1989, Irani and Schwartz 1990), where two major mast cell sub-classes have been identified.

However, a few SMCP-positive cells were observed in skin indicating proteinase heterogeneity in the dermal mast cell population. This observation may be analogous to the situation in man, where dermal CTMC can be divided into two sub-populations based on the presence or absence of a chymase (Schwartz 1989, Haas 1995). In the rat, in marked contrast to man, the chymase (RMCP-I) is pre-eminent, and it is the presence or absence of tryptase that delineates subpopulations of the cutaneous CTMC subset (Chen et al 1993). In the dog, in cutaneous mastocytoma cell lines, although chymase and tryptase can be asynchronously expressed, it is not known if this is a reflection of true heterogeneity within the cutaneous mast cell population, or if it is purely as a result of differences in the state of maturation of these neoplastic cells (Caughey et al 1988c). On the basis of cellular morphology, the anti-SMCP antibody did not appear to react with basophils. However, because of the difficulty in distinguishing basophils and mast cells in tissue sections and the apparent paucity of the former in tissues, the possibility that antibody to SMCP cross-reacts with basophils cannot be discounted.

The presence in the liver of a relatively large population of cells putatively identified as LGLs was noted. However, careful examination indicated that these cells did not contribute to the

toluidine blue-positive cell population in other tissues as stained cells were typically well-granulated and mononuclear suggesting a mast cell, rather than a basophil or lymphocyte lineage. Moreover, these tissues (with the exception of skin) had a high correlation between cell numbers detected with toluidine blue and with antibody to SMCP ($r^2 = 0.96$, $p<0.001$).

The presence of high numbers of thymic mast cells has been reported previously during studies in rats, although their role remains obscure (Huntley et al 1993). However, since mast cells produce a wide range of cytokines (Burd et al 1989, Gordon and Galli 1990a), it is tempting to speculate that mast cell-derived cytokines may modulate or augment T-cell responses. Certainly, the role and function of ovine thymic mast cells warrants further study.

Despite there being appreciable numbers of mast cells identified both histochemically and immunohistochemically in trachea, no SMCP could be detected in tracheal homogenates. It is possible that this indicates the presence of a distinct proteinase that is able to cross-react with the anti-SMCP antibody in paraformaldehyde fixed tissue sections, but is refractory to detection by the ELISA method. Alternatively, it is known that both serum and lymph contain "factors" that may interfere with SMCP - antibody interactions (Huntley et al 1987), these possibly being α_2- macroglobulin or serpins (Huntley 1991). It can be speculated that high inhibitor content in tracheal tissue may have prevented determination of SMCP by ELISA, although immunohistochemical identification in fixed tissue was still possible. Certainly, it is known that α_2-macroglobulin and α_1-proteinase inhibitor can block the detection of SMCP by the ELISA (Huntley 1991). This anomaly warrants further investigation, which could initially be undertaken by "spiking" the tissue homogenates with SMCP, and identifying the molecular weight of the SMCP-inhibitor complexes present on blots.

Previous studies have shown a correlation between the number of gastrointestinal mast cells detected with anti-SMCP and the tissue content of SMCP (Huntley 1991). On the basis of

these results, and the finding that this proteinase was released systemically into blood and lymph following nematode challenge in immune sheep (Huntley et al 1987), SMCP was considered a "MMC" enzyme and analogous to RMCP II in the rat (Gibson et al 1987). However, the present investigation suggests that SMCP is not unique to MMC, as although there are significant correlation's ($r^2 = 0.28$, $p<0.001$) between SMCP tissue content and mast cell numbers for the majority of tissues, the skin and liver are apparent exceptions.

The results of this study indicate that SMCP is present in all mast cells in most of the tissues studied, with the exception of skin. This is quite distinct from the situation in man and rodents, where chymase is the predominant mast cell protease in skin. These ovine dermal mast cells presumably contain a proteinase which is antigenically distinct from SMCP and the sheep is, therefore, similar to other species studied in that there is heterogeneity of granule proteases. However, there are also species differences which may have important implications in terms of function and activity of these cells. For instance, in the rat, RMCP-I-containing CTMC predominate in a variety of connective tissues including skin and are widely distributed throughout these organs. Although RMCP-II - containing MMC are found principally in the gastrointestinal tract they are not confined to this tissue, and indeed can be located in non-mucosal sites (Huntley et al 1993). The distribution of ovine mast cell subsets is apparently different to the rat, with clearly defined anatomical locations for the mast cell proteinase populations. By inference from data in other species a number of granule proteinases have yet to be identified. For example, a second chymase, related to but distinct from SMCP, may be present in ovine mast cells (Huntley 1991). Secondly, ovine mast cell tryptase has not, as yet, been isolated. (Interestingly, tryptase has recently been demonstrated both histochemically and immunohistochemically in bovine cutaneous mast cells (Welle et al 1995)). Once these two enzymes have been isolated and their distribution determined, it will be possible to further define ovine mast cell subsets. From a functional standpoint it is

important to determine whether chymase (as in rats and mice) or tryptase (as in the dog and man) is the more functionally significant. (Nadel 1991). As SMCP is present in the majority of ovine mast cells, it may be the more important of the two enzymes. However, the recent unexpected finding that SMCP also possesses trypsin-like activity (Pemberton, Huntley and Miller, *submitted*) may make such a simplistic classification impractical. The tryptic activity of SMCP is manifest by the cleavage of bonds in bovine albumin with a lysine at P_1 and aspartate or glutamate (acidic residues) at P_4 and P_4', a structure that occurs frequently in ovine albumin and which suggests that this could act as a native substrate for SMCP. Indeed, apart from this dual activity (as both a tryptase and chymase), SMCP is very similar to human and rodent chymases in terms of the relative effect of inhibitors (e.g. α_1-PI) and the possession of similar N-terminal amino acid sequences (Pemberton, Huntley and Miller, *submitted*).

The findings of this study could be further clarified by the use of ovine anti-IgE or ovine anti-*c-kit*. However, these reagents are currently not available for use in the sheep.

This investigation has defined the tissue distribution of SMCP, and established that mast cell heterogeneity, based on differences in proteinase expression, occurs in the sheep. Apparent proteinase heterogeneity within the ovine dermal mast cell population has also been identified. Diversity appears to exist between species in mast cell proteinase heterogeneity and expression, and further studies involving the isolation of ovine dermal mast cell proteinase(s) are required for a full evaluation of mast cell heterogeneity in the sheep.

Given that SMCP was shown to be present in a proportion of ovine dermal mast cells, further investigation of the effects of SMCP and of putative mast cell secretagogues in ovine skin *in vivo* (Chapters 4 and 5) and on recombinant ovine interleukin-3-dependent bone marrow-derived mast cells (rOv.IL-3 BMMC) *in vitro* (Chapter 7) is warranted.

CHAPTER 4

CUTANEOUS RESPONSES TO SUBSTANCE P, COMPOUND 48/80 AND CALCIUM IONOPHORE A23187 : THEIR IDENTIFICATION AS PUTATIVE OVINE DERMAL MAST CELL SECRETAGOGUES.

4.1. Introduction

Given that the results from experiment 3.1 suggested that ovine dermal mast cells were distinct from mast cells at other sites and that, within the dermis, two populations of mast cells could be identified based on their content of granule chymase, it was logical to investigate the inflammatory response evoked by activation of the dermal mast cell population. It is known that mast cells in rodents and in man are functionally heterogeneous in their responses to activation and degranulation by chemical secretagogues. For example, rat cutaneous mast cells are sensitive to the neuropeptide substance P (sP) and the polybasic compound 48/80 (48/80), whereas rat intestinal mast cells are relatively insensitive to these agents (Enerbäck 1966c, Galli 1990). Likewise, human cutaneous mast cells are responsive to sP, 48/80 and the calcium ionophore A23187 (A23187), whereas mast cells from lung and colon are insensitive to the actions of both sP and 48/80 (Lowman et al 1988). In mice, sP and 48/80 evoke neutrophil infiltration in skin as a consequence of mast cell degranulation (Tomoe et al 1992). Furthermore, since bovine cutaneous mast cells release histamine in response to sP and A23187 (Hunt et al 1991), it is likely that ovine cutaneous mast cells respond to the same secretagogues. Indeed, several studies have reported that cutaneous mast cells in a variety of species can be activated by sP, 48/80 and A23187 (Table 4.1). Therefore, the responses of ovine dermal mast cells to these secretagogues could be compared with those in other species, which would extend our knowledge of the phenotypic heterogeneity of the ovine dermal mast cell population. Likewise, these *in vivo* responses could be similarly compared to those of rOv.IL-3 BMMC generated in culture (Chapters 6 and 7). Although cytotoxic and known to affect a wide range of cell types, A23187 was used in these studies as it was the only secretagogue that had previously been used to activate an ovine mast cell population (Huntley et al 1992). This therefore provided a baseline of response against which other secretagogues could be assessed. The overall aim of the experiments described in this chapter

was, therefore, to determine if sP, 48/80 and A23187 were secretagogues for ovine cutaneous mast cells.

	Substance P (sP)	Compound 48/80 (48/80)	Calcium ionophore A23187 (A23187)
Man	Benyon, Lowman and Church 1987 Ebertz et al 1987 Foreman 1987 Lawrence et al 1987 Lowman, Benyon and Church 1988 Lowman et al 1988 Cohan et al 1989 Tainsh et al 1991	Benyon, Lowman and Church 1987 Lawrence et al 1987 Tainsh et al 1991	Benyon et al 1986 Benyon, Lowman and Church 1987 Benyon et al 1987 Lowman et al 1988 Kaminer et al 1991 Tainsh et al 1991 Goldstein et al 1992 Tainsh et al 1992
Rat	Brain and Williams 1988 Galli 1990	Pearce 1986	Barrett, Ali and Pearce 1985
Mouse	Matsuda et al 1989 Yano et al 1989 Tomoe et al 1992	Matsuda et al 1989 Tomoe et al 1992	
Cow	Hunt et al 1991		Hunt et al 1991
Gerbil		Nawa et al 1994	

Table 4.1. Studies in various species reporting cutaneous mast cell activation by sP, 48/80 and A23187.

4.2. Experiment 4.1 - Assessment Of Mast Cell Numbers In Flank Skin

4.2.1. Experimental Aim and Design

The aim of the investigation was to determine whether there were differences in the regional distribution of mast cells within the area of the flank chosen for intradermal skin testing (Fig. 2.5). This information is necessary so that subsequent measurements of the magnitude of cutaneous response following intradermal challenge with mast cell secretagogues could be evaluated in relation to cutaneous mast cell density.

At post-mortem examination, the left and right flanks of four Blackface ewes were clipped and prepared as described (2.5.2.1). Samples of skin were removed from the cranial, caudal and ventral margins of each prepared area of flank using a 6mm skin biopsy punch. Additionally, further samples were taken from a sparsely haired region in the groin (both left and right sides). Samples were fixed and processed (2.4.1), sections being stained with toluidine blue pH 0.5 (2.3.9). The number of mast cells per mm^2 skin for each section was determined as described (2.5.3), the eight regions being compared statistically for significant differences between individual groups by the use of one-way analysis of variance (ANOVA).

4.2.2. Results

The sample means and standard deviations (n = 4 for each group) are shown for the eight regions (Fig. 4.1). One-way ANOVA indicated no significant difference between groups ($P = 0.22$). This indicated that within the site chosen on the flank there was unlikely to be any marked variation in mast cell distribution and thus it was warranted to use this site for intradermal challenge with mast cell secretagogues.

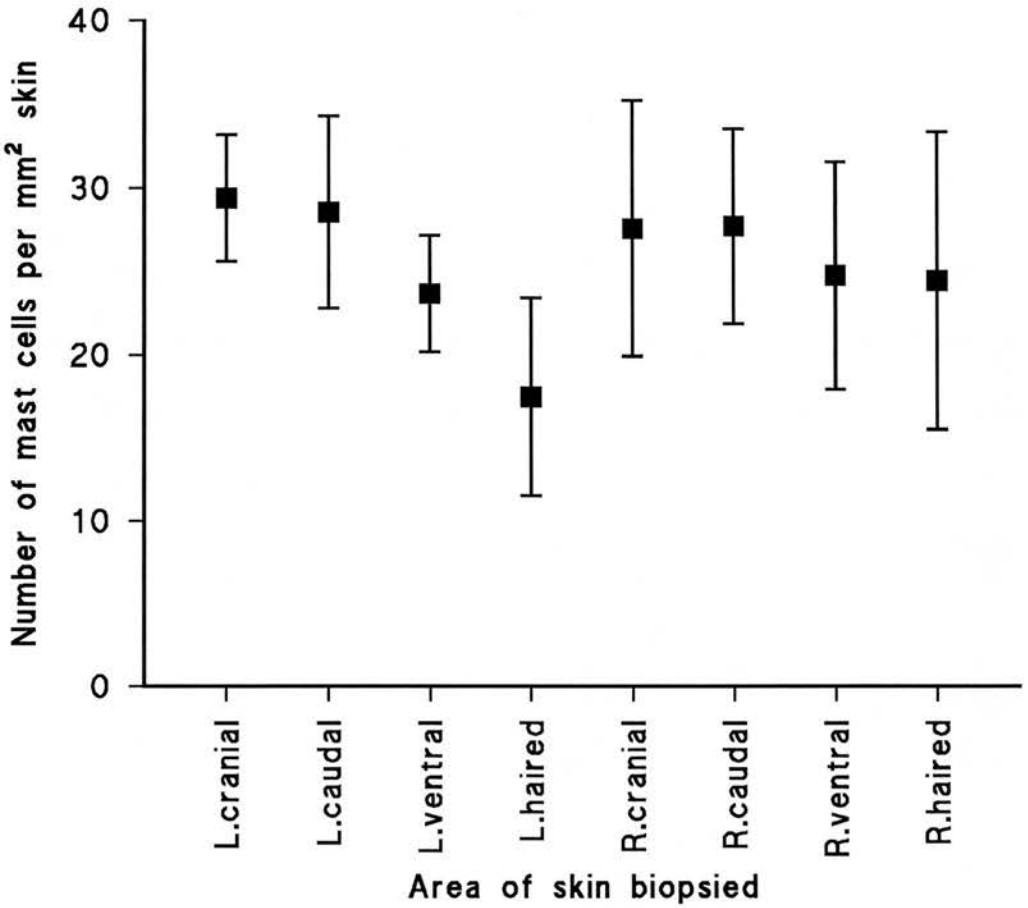


Figure 4.1. Histological assessment of mast cell numbers in toluidine blue stained sections from three sites on both flanks (from within the flank area as shown in Figure 2.4; left and right cranial flank, caudal flank and ventral flank) and from an area of sparsely haired skin in the groin (left and right haired). Datapoints depict the sample mean and error bars the standard deviation for each group of measurements. One-way analysis of variance (ANOVA) indicated no statistically significant difference between the individual groups (P = 0.21).

4.3. Experiment 4.2 - Cutaneous Responses To Substance P (sP), Compound 48/80 (48/80) And Calcium Ionophore A23187 (A23187) In The Sheep

4.3.1. Experimental Aim and Design

The aims of this study were to determine if sP, 48/80 and A23187 could evoke a response in ovine skin *in vivo* and, if so, to determine the optimal concentration of each agent required. The cutaneous response was assessed by measurement of weal volume and by quantifying the cutaneous mast cell and inflammatory cell changes histologically.

Twelve cast Blackface ewes were used in the study. These had been grazed at pasture and had not had any anthelmintic treatment immediately prior to investigation. The group had been treated once with a topical ectoparasiticide to remove lice and other ectoparasites (deltamethtrin 1% $^{w}/_{v}$, Spot On Insecticide, Coopers®; Pitman-Moore Ltd., Crewe, UK) before being housed, and once while housed.

4.3.1.1. Study protocol

The study was divided into two stages. In the first stage three separate dose response studies were undertaken to assess whether each of the putative secretagogues could produce a weal response in ovine skin and, if so, what the optimal dose of each agent was. Histological examination of skin biopsies was undertaken for evidence of cellular infiltration and mast cell degranulation. In the second stage three separate time course studies were performed using the optimal dose of each agent (based on weal volume data from the first, dose response, stage if a response had been elicited).

The initial dose range of each agent used (outlined in 2.5.1.1) was derived from published data for their use in other species (sP : Lawrence et al 1987, Yano et al 1989, Iwamoto et al 1992; 48/80 : Lawrence et al 1987, Wahlgren, Hagermark and Bergstrom 1991, Tomoe et al 1992; A23187 : Hunt et al 1991, Huntley et al 1992). Intradermal skin testing, weal volume

measurement and skin punch biopsy was carried out on flank skin as described in 2.5.2, all measurements being made blind with respect to the experimental protocol.

4.3.1.2. Dose response studies

Each of the dose response studies (sP, 48/80 and A23187) was performed on the left flank of four sheep. A biopsy of untreated skin was taken from one of the six grid sites immediately prior to the start of the study (untreated control). Four of the remaining five sites were used to assess different concentrations of the agent under investigation, the final site being used to assess the appropriate diluent control. Weal volume was measured at each of the injection sites at 5, 10, 15 (or 20), 60, 180 and 360 minutes post-injection. A skin biopsy was taken from each of these five sites at 360 minutes post-injection.

4.3.1.3. Time course studies

Time course studies were performed on the right flank of four sheep. A biopsy of untreated skin was taken from one of the six grid sites immediately prior to the start of the study. The optimal dose of the agent was injected intradermally at four of the remaining sites, the final site being used to assess the relevant diluent control. Biopsies were taken at 15, 60, 180 and 360 minutes after injection of the agent. Biopsy of the diluent control was undertaken at the peak of the weal response, as determined from the dose response study.

Mast cell (toluidine blue pH 0.5), neutrophil (haematoxylin and eosin) and eosinophil (carbol chromatrope) cell counts on stained skin sections were undertaken (2.5.3). The percentage of extensively degranulated mast cells on toluidine blue-stained sections was also calculated (2.5.4).

The weal volume for each agent at each datapoint was compared against the appropriate diluent control by use of the Mann-Whitney U test (2.5.7). The number of mast cells, neutrophils, eosinophils and the percentage of extensively degranulated mast cells in skin

sections for both the dose response and time course studies were compared against appropriate diluent controls by use of the Mann-Whitney U test (2.5.7).

4.3.2. Results

4.3.2.1. Weal Responses

A significant weal response was evoked by the intradermal injection of 50µl of 10^{-5} M substance P (sP) (Fig. 4.2 (a); $P<0.05$), but was not seen with the other concentrations of sP tested. When 10^{-5} M sP was injected in a time course study the weal response was significant at 10 and 20 minutes post-injection (Figs. 4.3 (a) and 4.3 (b); $P<0.05$).

Compound 48/80 (48/80), despite being used at a wide range of concentrations (1µg/ml to 500µg/ml), failed to produce a statistically significant weal response (Fig. 4.2 (b)). Intradermal injection of 50µl of 250µg/ml 48/80 failed to evoke a statistically significant weal response at any time point during the time course study (Figs. 4.3 (c) and 4.3 (d)).

Intradermal injection of 50µl of 10^{-4} M A23187, but none of the other concentrations of A23187, evoked a significant weal response (Fig. 4.2 (c); $P<0.05$). Injection of 10^{-4} M A23187 produced a significant weal response at 5, 10, 20 and 60 minutes ($P<0.05$), the peak occurring at 60 minutes (Figs. 4.3 (e) and 4.3 (f)).

Using optimal concentrations of each agent, the volume of the peak weal response to A23187 (median 63.1 mm^3, range 36.9 mm^3 to 163.7 mm^3 at 60 minutes; Figs. 4.3 (e) and 4.3 (f)) exceeded that produced by sP (median 14.2 mm^3, range 11.2 mm^3 to 41.6 mm^3 at 20 minutes; Figs. 4.3 (a) and 4.3 (b)).

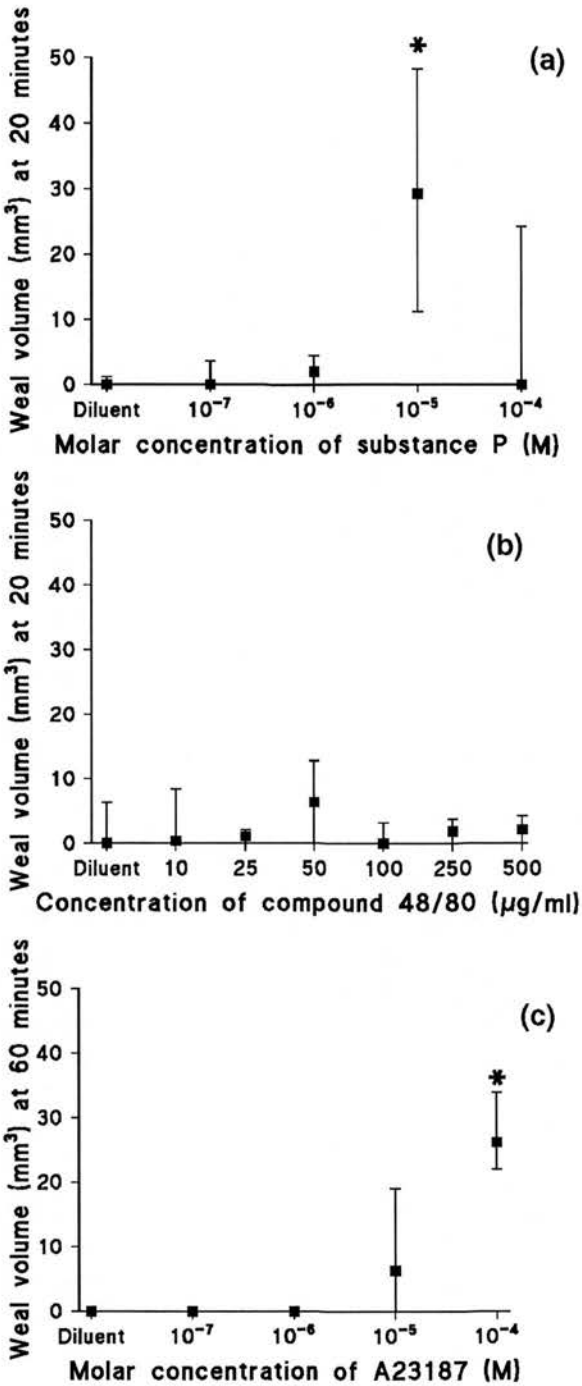


Figure 4.2. Dose response studies (weal volume against concentration) for sP **(a)**, 48/80 **(b)** and A23187 **(c)**. Datapoints depict the median and error bars demonstrate the range for each group of measurements. * represents statistical significance in comparison to the diluent control ($P<0.05$).

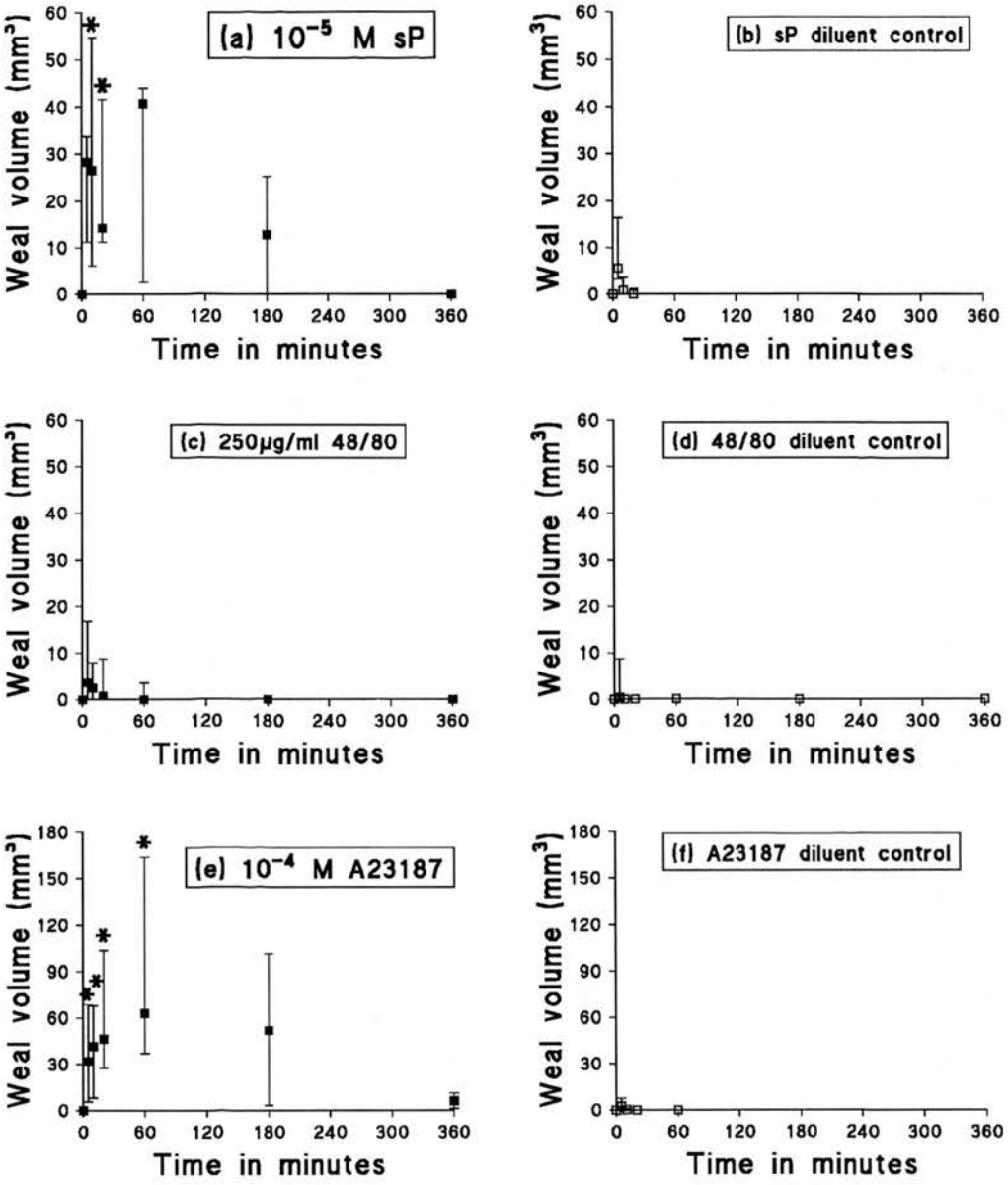


Figure 4.3. Time course studies (weal volume against time) for 10^{-5} M sP (a), 250µg/ml 48/80 (c), 10^{-4} M A23187 (e), and their respective diluent controls (b), (d), and (f). Datapoints depict the median and error bars demonstrate the range for each group of measurements. * represents statistical significance in comparison to diluent controls ($P<0.05$). Note that for sP and A23187 the diluent controls were biopsied at the time of maximal statistically significant weal response i.e. 15 minutes and 60 minutes respectively. Therefore, no weal volume measurements are available for each agents diluent control after these time-points.

4.3.2.2. Histology

For the dose response studies, all biopsy samples (except for those controls from time zero, untreated skin) were taken at 360 minutes. As it was not known prior to the experiment when the maximum histological change for each agent would occur, and because there were limitations on the number of biopsies available from each animal, in time course studies the biopsy of the respective diluent control was taken at the time of the maximal weal response (i.e. sP 15 minutes, A23187 60 minutes). In the case of 48/80, where no weal response had been evoked, the diluent control was biopsied at 360 minutes. Therefore, the biopsy of the diluent control coincided with the time of maximal weal response where present, but not necessarily with the time of maximal histological change.

4.3.2.2.1. Mast cells

There was no statistically significant effect of sP, 48/80 or A23187 on the absolute number of mast cells in either the dose response or time course studies (Figs. 4.4 (a) to 4.4 (f)). In the dose response study a significant increase in the percentage of extensively degranulated mast cells occurred with 10^{-5} M sP, but not with the other concentrations of sP used, in comparison to the diluent control at 360 minutes (Figs. 4.5 (a) and 4.6). This statistically significant increase ($P<0.05$) was first demonstrable by 15 minutes post-injection and remained constant thereafter (Fig. 4.5 (b)).

An apparent increase in the percentage of extensively degranulated mast cells was seen with all of the concentrations of 48/80 used in the dose response study (Fig. 4.5 (c)). However, these changes were not statistically significant. Owing to the absence of a demonstrable weal response to the initial injections of 48/80, in total a range of six concentrations were used in the four sheep involved in the dose response study. This resulted in there being fewer individual biopsies ($n = 2$ or 3) for each concentration of 48/80 used. As a result, owing to reduced sample group size, statistical significance was not attained, although the trend was

apparent. However, in the time course study, four biopsies were available for each of the diluent control and 48/80 challenged sites at 360 minutes post-injection. Thus, in this study, a significant increase ($P<0.05$) in the percentage of extensively degranulated mast cells was noted at this time point (Fig. 4.5 (d)). Although diluent controls were not available for the 15, 60 and 180 minutes post-injection 48/80-challenged biopsies, the degree of mast cell degranulation in these is of the same magnitude as at 360 minutes post-injection.

Only the highest concentration of A23187 (10^{-4} M) produced a significant increase in the percentage of extensively degranulated mast cells in comparison to the diluent control at 360 minutes post-injection (Fig. 4.5 (e); $P<0.05$). From the time course study, this significant effect had occurred by 60 minutes post-injection (Fig. 4.5 (f)).

4.3.2.2.1. Neutrophils

Neutrophil influx was evoked by intradermal injection of 50µl of 10^{-5} M sP, in comparison to the diluent control, by 360 minutes post-injection (Fig. 4.7 (a); $P<0.05$). In the time course study, no significant neutrophil influx had occurred by 15 minutes post-injection (time of the maximal weal response for sP) (Fig. 4.7 (b)). The neutrophil influx increased up to 180 minutes post-injection, and receded thereafter (Fig. 4.7 (b)).

Compound 48/80 evoked neutrophil influx in an apparently dose-responsive fashion (Fig. 4.7 (c)), but again, as a consequence of reduced group size owing to the wide range of 48/80 concentrations used in the dose response study, statistical significance was not attained. Intradermal injection of 50µl of 250µg/ml 48/80 in the time course study evoked significant neutrophil influx in comparison to the diluent control at 360 minutes post-injection (Fig. 4.7 (d); $P<0.05$). Neutrophil influx was still increasing between 180 and 360 minutes (Fig. 4.7 (d)).

Intradermal injection of 50µl of 10^{-4} M A23187 evoked significant neutrophil influx at 360 minutes post-injection in the dose response study (Fig. 4.7 (e)). This was not significant at 60 minutes (Fig. 4.7 (f)), the time of the peak weal response of A23187. Neutrophil influx still appeared to be increasing between 180 and 360 minutes post-injection (Fig. 4.7 (f)).

The initial pattern of neutrophil influx was the same for all three agents. By 15 minutes post-injection there was congestion of deep dermal vessels with the occasional neutrophil present within the lumen (Fig. 4.8). Margination of neutrophils was obvious by 60 minutes post-injection (Fig. 4.9), neutrophil emigration being underway. Marked dermal oedema was present. By 180 minutes post-injection a dense neutrophilic infiltrate had developed (Fig. 4.10).

In assessing the diluent injected sites at six hours post-injection in comparison to the time zero control sites (T0), significant neutrophil influx ($P<0.05$) was seen in both the sP and A23187 diluent control treated sections (median 69.8 neutrophils mm^{-2} , range 37.7 to 73.4 neutrophils mm^{-2} [sP diluent] versus median 13.6 neutrophils mm^{-2} , range 10.6 to 13.9 neutrophils mm^{-2} [time zero control] and median 21.5 neutrophils mm^{-2}, range 14.6 to 31.1 neutrophils mm^{-2} [A23187 diluent] versus median 5.3 neutrophils mm^{-2}, range 0.7 to 9.3 neutrophils mm^{-2} [time zero control]).

4.3.2.2.3. Eosinophils

Substance P failed to evoke statistically significant eosinophil influx in either the dose response or time course studies (Fig. 4.11 (a) and (b)).

Compound 48/80 appeared to evoke eosinophil influx in a dose-responsive manner (Fig. 4.11 (c)) but again, owing to reduced group size, statistical significance was not attained. However, in the time course study, the injection of 50µl of 250µg/ml 48/80 resulted

in statistically significant eosinophil influx at 360 minutes post-injection (Fig. 4.11 (d), $P<0.05$).

As with neutrophil influx, only the intradermal injection of 50µl of 10^{-4} M A23187 evoked statistically significant eosinophil influx (Fig. 4.11 (e), $P<0.05$; Fig. 4.12).This effect was not apparent by 60 minutes post-injection in the time course study (Fig. 4.11 (f)).

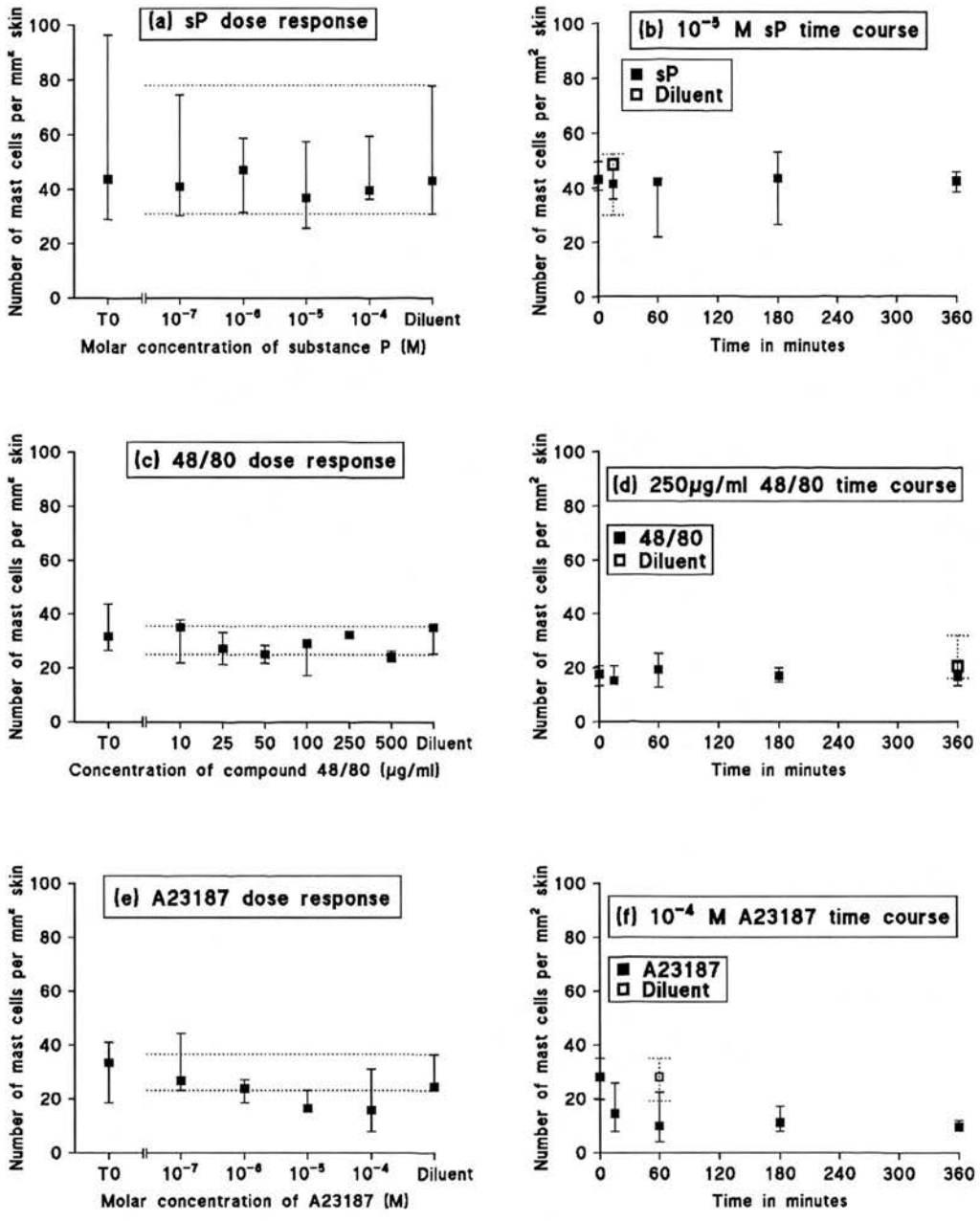


Figure 4.4. Histological assessment of mast cell numbers in toluidine blue stained sections. Dose response studies at 360 minutes (mast cell number against concentration) are shown for sP (a), 48/80 (c) and A23187 (e). Time course studies (mast cell number against time) are shown for 10^{-5} M sP (b), 250µg/ml 48/80 (d) and 10^{-4} M A23187 (f). T0 represents measurements for time zero, untreated skin. Datapoints depict the median and error bars demonstrate the range for each group of measurements. Dotted lines represent the range for each group of measurements for the respective diluent control.

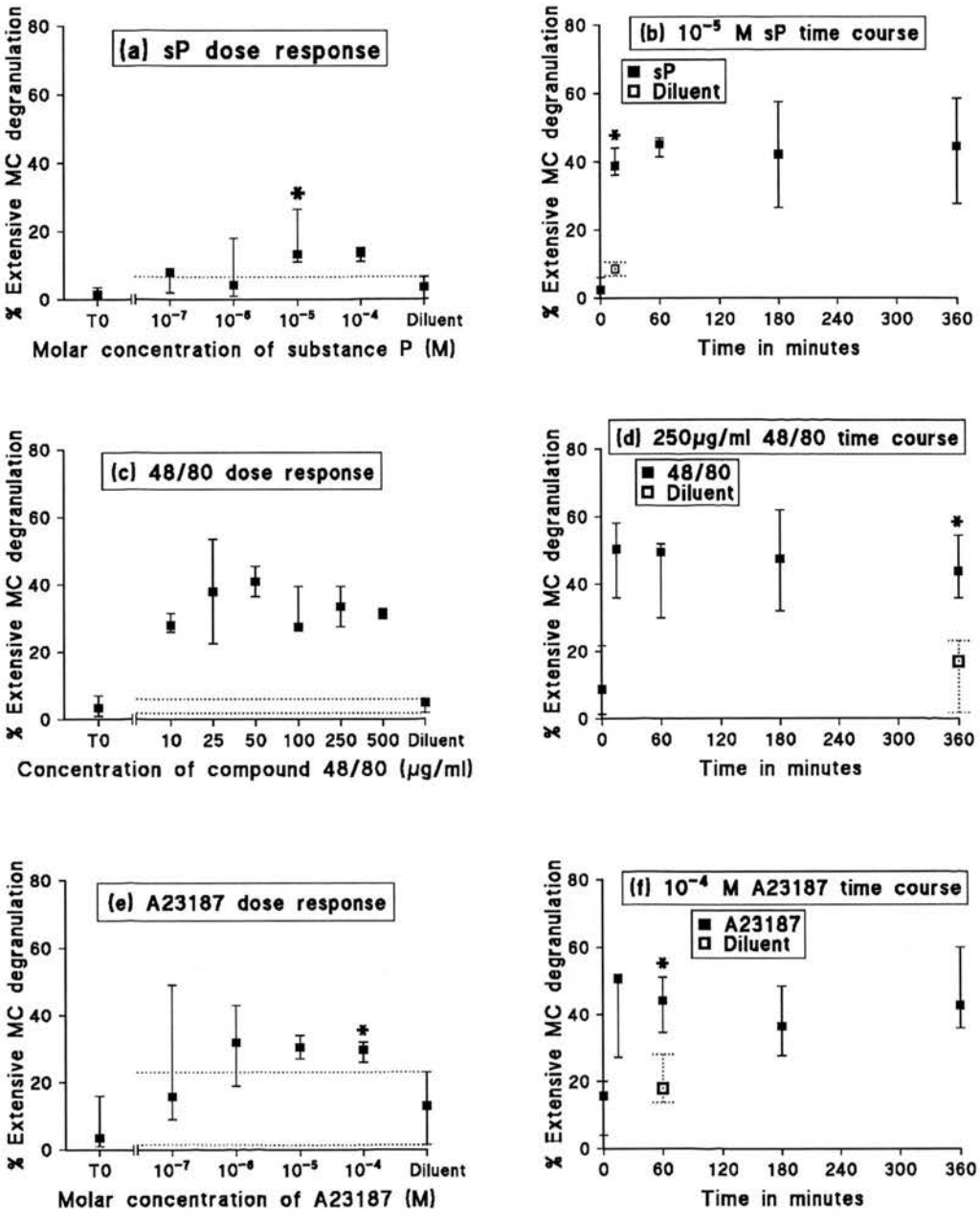


Figure 4.5. Histological assessment of percentage of extensively degranulated mast cells in toluidine blue stained sections. Dose response studies at 360 minutes (percentage extensive degranulation against concentration) are shown for sP (a), 48/80 (c) and A23187 (e). Time course studies (percentage extensive degranulation against time) are shown for 10^{-5} M sP (b), 250µg/ml 48/80 (d) and 10^{-4} M A23187 (f). T0 represents measurements for time zero, untreated skin. Datapoints depict the median and error bars demonstrate the range for each group of measurements. Dotted lines represent the range for each group of measurements for the respective diluent control. * represents statistical significance in comparison to the appropriate diluent control ($P<0.05$).

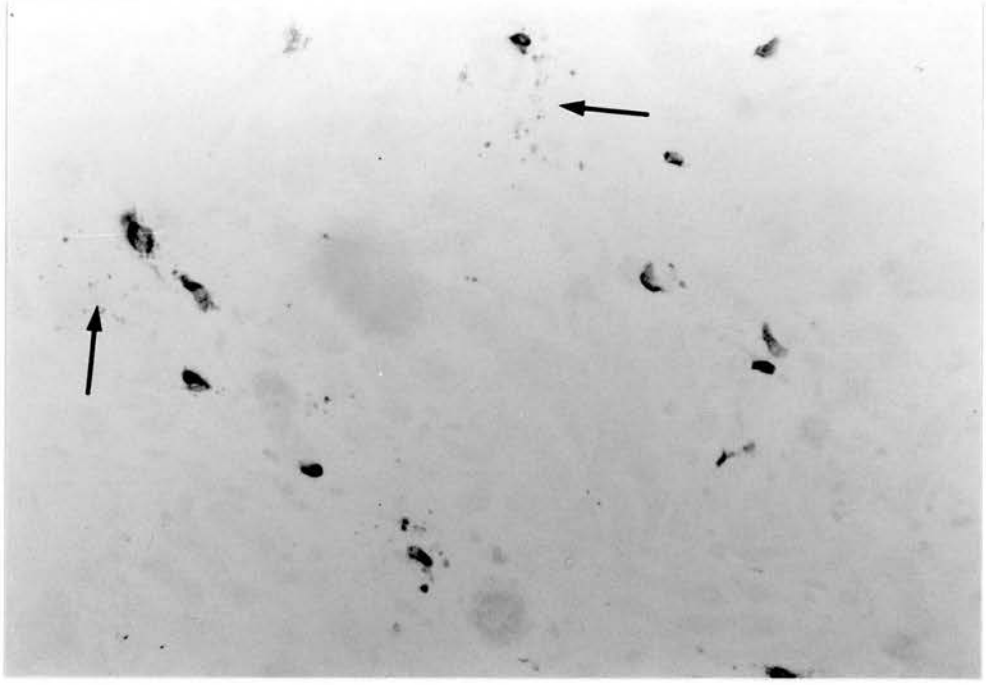


Figure 4.6. Degranulation of dermal mast cells in substance P treated section (biopsy taken at 15 minutes post-injection). Mast cell granules can be seen free in the dermis (arrowed). (Toluidine blue x 500).

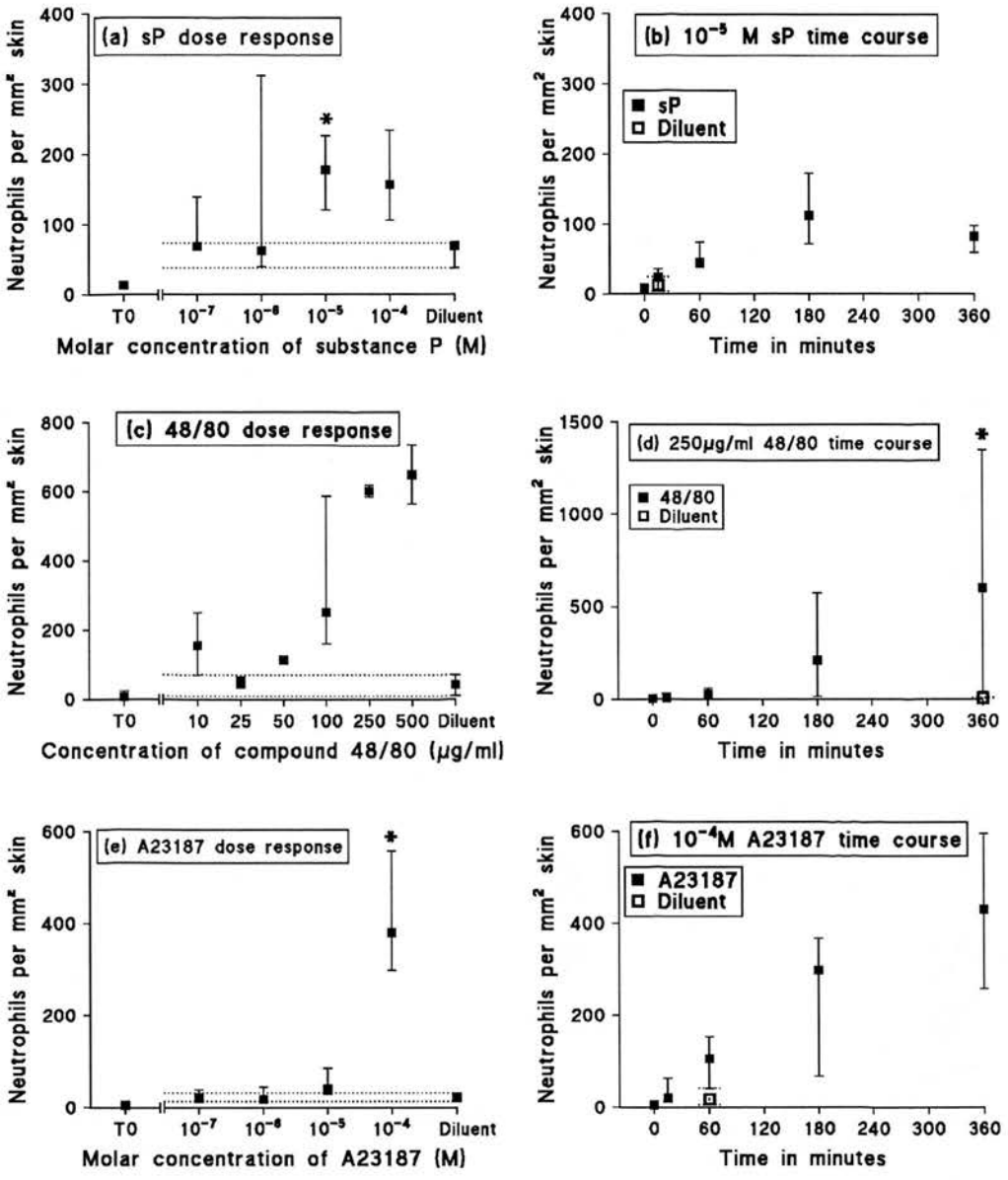


Figure 4.7. Histological assessment of dermal neutrophil influx in Haematoxylin and Eosin stained sections. Dose response studies at 360 minutes (number of neutrophils against concentration) are shown for sP (a), 48/80 (c) and A23187 (e). Time course studies (number of neutrophils against time) are shown for 10^{-5} M sP (b), 250µg/ml 48/80 (d) and 10^{-4} M A23187 (f). T0 represents measurements for time zero, untreated skin. Datapoints depict the median and error bars demonstrate the range for each group of measurements. Dotted lines represent the range for each group of measurements for the respective diluent control.
* represents statistical significance in comparison to the diluent control ($P<0.05$).

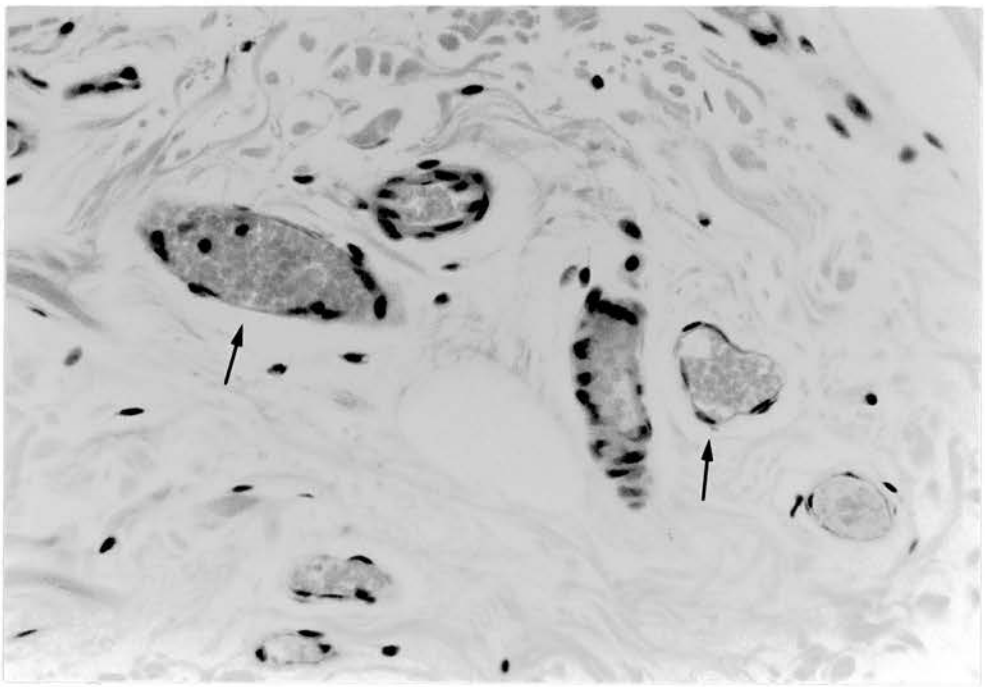


Figure 4.8. Congestion of dermal vessels (arrowed) in response to intradermal injection of 50µl of 10^{-4} M substance P at 15 minutes post-injection. Neutrophils were occasionally observed in the lumen. (H & E x 500).

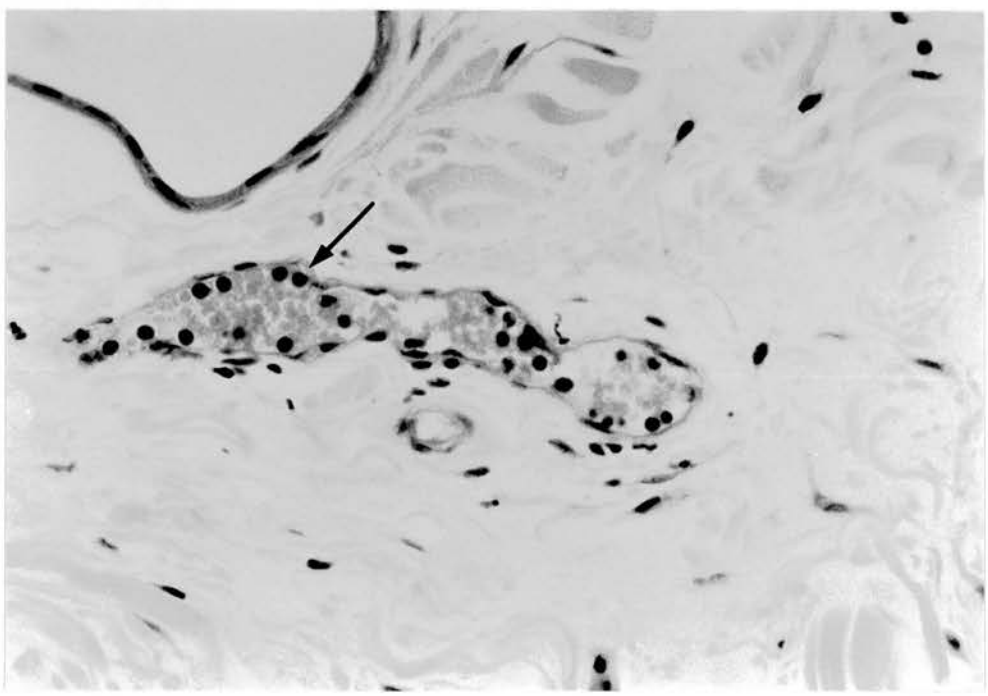


Figure 4.9. Margination of neutrophils (arrowed) in dermal vessels at 60 minutes post-injection in response to intradermal injection of 50µl of 10^{-4} M substance P. (H & E x 500).

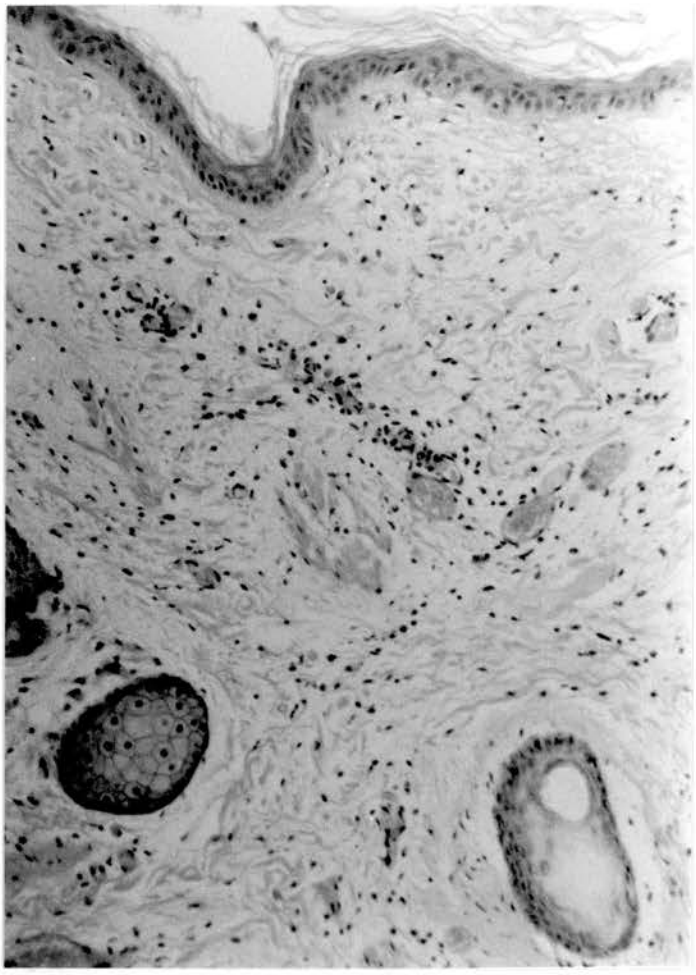


Figure 4.10. Neutrophilic dermal infiltrate at 180 minutes post-injection in response to intradermal injection of 50µl of 10^{-4} M substance P. (H & E x125).

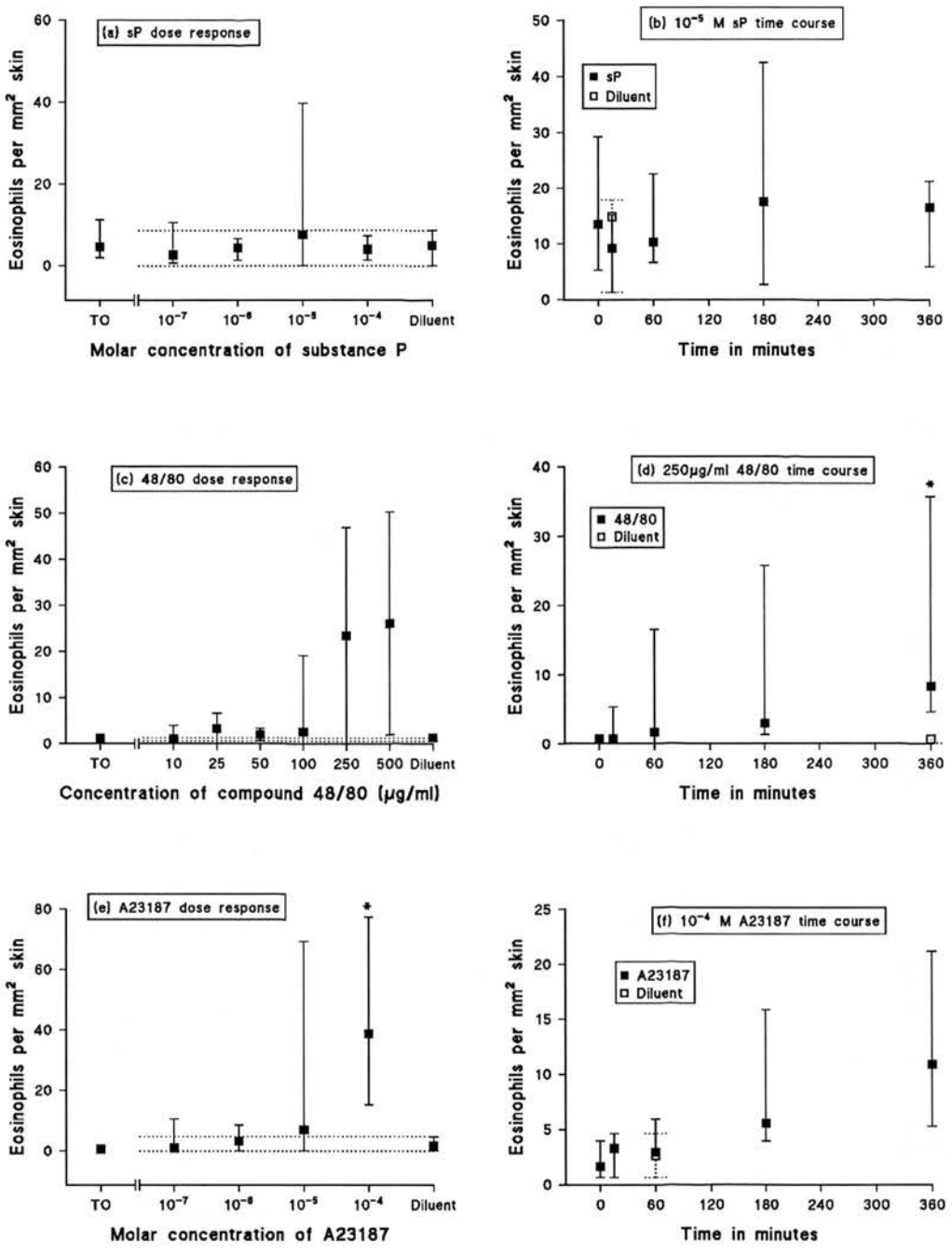


Figure 4.11. Histological assessment of dermal eosinophil influx in carbol chromotrope stained sections. Dose response studies at 360 minutes (number of eosinophils against concentration) are shown for sP (a), 48/80 (c) and A23187 (e). Time course studies (number of eosinophils against time) are shown for 10^{-5} M sP (b), 250µg/ml 48/80 (d) and 10^{-4} M A23187 (f). T0 represents measurements for time zero, untreated skin. Datapoints depict the median and error bars demonstrate the range for each group of measurements. Dotted lines represent the range for each group of measurements for the respective diluent control.
* represents statistical significance in comparison to the diluent control ($P<0.05$).

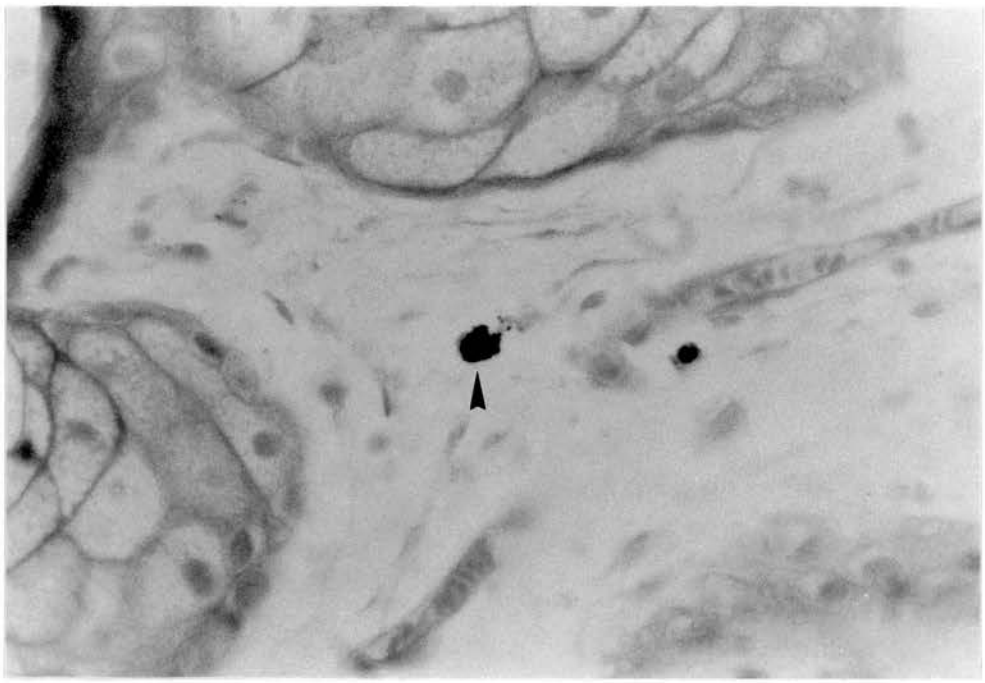


Figure 4.12. Eosinophil in A23187 treated section (10^{-4} M A23187, biopsy taken at 360 minutes post-injection). (Carbol chromotrope x 500).

4.4. Discussion

The purpose of this study was to determine the effects of known mast cell secretagogues on ovine dermal mast cells and to quantify the weal responses and cellular infiltration in the skin post-challenge. In man, the characteristic finding in response to histamine injection or to positive intradermal allergen testing is of an immediate weal and flare reaction, the weal being considered a more accurate estimator of response in comparison to the flare (Champion and Burton 1992). Given that mast cell degranulation had occurred in response to each of the three agents, the dominant feature in the sheep was that of a weal, with no flare response being noted. The lack of a consistent flare response is also a feature in the dog, where weal production is prominent (Sture et al 1995). It is known, however, that ovine mast cells do contain histamine (200ng/10^6 MMC, 48ng/10^6 CM-BMMC; Huntley et al 1992) and can generate LTC_4 (5.3ng per 10^6 CM-BMMC, in response to A23187; Huntley et al 1992). The results indicate that sP, 48/80 and A23187, used at optimal concentrations and assessed at appropriate time points, can evoke statistically significant mast cell degranulation and neutrophil influx *in vivo*. Of these agents, only sP and A23187 were capable of evoking a statistically significant weal response in ovine skin, and none of these three agents altered the absolute number of mast cells present at challenged sites.

The weal response to (sP) appeared to be subject to a "window" effect, only 10^{-5} M sP producing a statistically significant response. This is in contrast to the dose-dependant effect observed in man (Coulson and Holden 1990, Gianetti and Girolomani 1989). It is interesting that the only statistically significant neutrophil influx was also seen to occur with this concentration of sP. This "window" effect may be responsible for the failure to demonstrate plasma leakage following intradermal injection of sP in sheep (Colditz 1991b) as, correcting for the volume per injection site, the optimal amount of sP injected in the present study was 1000 times greater than the maximum injected in the plasma leakage study. The presence of a

statistically significant weal response at 10 and 20 minutes (maximal mean weal response) for sP was consistent with the occurrence of maximal weal volume in man (Coulson and Holden 1990, Gianetti and Girolomani 1989). The present study has also shown that sP evokes neutrophil influx in ovine skin, which is in agreement with studies in the mouse (Yano et al 1989, Matsuda et al 1989, Tomoe et al 1992). However, unlike the mouse, significant eosinophil influx was not evoked (Yano et al 1989, Matsuda et al 1989, Tomoe et al 1992). Furthermore, sP-induced neutrophil recruitment is mast cell-dependant (Matsuda et al 1989, Yano et al 1989), and has been shown to be due to the N-terminal portion of sP (Iwamoto et al 1992). Statistically significant mast cell degranulation occurred by 15 minutes and neutrophil infiltration was maximal at 3 hours in ovine skin, although it is not known if this response is due to a direct effect of sP on mast cells. Substance P is a basic, positively charged peptide which may activate mast cells in a non-receptor-dependent process (Mousli et al 1994). It has been shown in the mouse that mast cells contain pre-formed stored TNF-α which, by activation through IgE-dependant or -independent mechanisms, can be immediately released (Gordon and Galli 1990a, Gordon and Galli 1991), although it is not known currently if ovine mast cells contain TNF-α. As TNF-α can promote leukocyte infiltration through effects on vascular endothelial cells (Pasyk and Cherry 1990), this represents one possible mechanism for the neutrophil influx observed in the present study. Indeed, it is known that TNF-α-stimulated endothelial cells express endothelial leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) that can mediate the subsequent adhesion of neutrophils and eosinophils (Kyan Aung et al 1991). Furthermore, human dermal mast cells have been shown to contain and release (upon activation) TNF-α, which directly induces ELAM-1 expression on dermal vascular endothelium (Walsh et al 1991) and, additionally, intradermal injection of TNF-α evokes neutrophil infiltration in ovine skin (Colditz and Watson 1992). Substance P reactivity is present in ovine skin (Nitsos and Rees 1993), and

thus the interaction between sP present in C nerve fibres (Foreman 1987) and mast cells could play a role in neurogenic inflammation (as is believed to occur in man) through cutaneous axon reflex vasodilation (Foreman 1993). Imbalances in sP and other neuropeptides have been implicated in the pathogenesis of human atopic disease (Gianetti et al 1992, Alabadie et al 1994) and psoriasis (Pincelli et al 1994). Mast cell chymases may help regulate cutaneous neurogenic inflammation (Caughey et al 1988d, Naukkarinen et al 1994) and thus modulate these disorders. Having demonstrated that ovine dermal mast cells also contain chymase (Chapter 3), it may indicate that similar regulatory mechanisms may occur in the sheep and this therefore provides a strong impetus for determining the mode of action and relationship between sP and the ovine cutaneous mast cell.

The lack of development of a weal response but with the presence of statistically significant mast cell degranulation, apparently dose-dependant neutrophil influx and statistically significant eosinophil influx with 48/80 was surprising, since the induction of a weal response to the agent in sheep has been previously reported (Lucio et al 1992). Given that 48/80 activates mast cells selectively (Foreman 1993) in a non-cytotoxic process (Johnson and Moran 1969), that it does not activate vascular endothelial cells (Tomoe et al 1992), and is known to activate cutaneous mast cells in other species (Table 4.1), the supposition would be that mast cell activation has played a role, at some stage, in the neutrophil and eosinophil influx. Certainly, *in vitro*, it has been demonstrated that 48/80-induced dermal mast cell degranulation can induce ELAM-1 expression (Klein et al 1989). The lack of a weal response could be dose-related, with the concentrations used not producing a "bolus" of histamine release, thereby preventing weal formation but inducing mast cell activation and mast cell cytokine induction (Gordon, Burd and Galli 1990) as part of the acute inflammatory response of which neutrophil infiltration is a part. It may be that the generation of a weal response requires accessory pathways involving activation of other cell types by mast cell-derived

mediators (e.g. leukotrienes), which are not generated during the cutaneous response to the intradermal injection of 48/80. Indeed, there are known species differences in the response to 48/80, as, for instance, much higher doses are required to produce histamine release in man in comparison to the rat (Lichtenstein et al 1979). It is conceivable that the lack of a weal response to 48/80 could be a feature of the breed of sheep used, but the presence of weal responses to other agents tends to reduce this possibility. Interestingly, bovine skin mast cells failed to release histamine in response to 48/80 (Hunt et al 1991).

Only the highest concentration of A23187 (10^{-4} M) gave a statistically significant weal response and neutrophil and eosinophil influx after intradermal injection. Higher concentrations of A23187 may be required to establish if a dose-dependant response is indeed occurring. In addition to the activation of human, rat and bovine cutaneous mast cells (Table 4.1), A23187 has been shown to activate ovine bone-marrow derived mast cells, cultured in the presence of lymphocyte-conditioned medium (Huntley et al 1992). Given that these cells are believed to represent a mixed population of ovine mast cells of both the mucosal and connective tissue phenotypes (Huntley et al 1992), it would suggest that at least one, or both, of the ovine mast cell populations is responsive to A23187. Although statistically significant mast cell degranulation had occurred by 60 minutes after A23187 injection, it cannot be concluded that this, and the neutrophil and eosinophil influx, was due to the direct stimulation of cutaneous mast cells. However, A23187 can presently be considered to be a putative ovine cutaneous mast cell secretagogue.

The neutrophil influx observed with the use of two of the diluents was not unexpected. In addition to the mechanical disruption upon injection, the need to initially dissolve A23187 in ethanol (and therefore produce an equivalent diluent) was unavoidable. Further studies to identify the most suitable diluents for the secretagogues may be justified. Ideally, a diluent control should be obtained for each time point in the time course studies. However, this was not feasible for ethical reasons and therefore the time at which weal response was maximal was chosen to assess the diluent control for each agent. It is essential when choosing diluents to use those that are pyrogen-free (Colditz 1991b) and contain minimal endotoxin since the latter has potent inflammatory potential in skin (Colditz and Movat 1984).

Both 48/80 and A23187 treated sites showed increased neutrophil influx from 180 to 360 minutes whereas neutrophil numbers in sP treated sites fell during this period. This represents a fundamental difference in the kinetics of the inflammatory response to these agents, and would justify further studies to investigate these responses by analysing the trafficking of cells through the dermis into afferent lymph.

All three agents evoked extensive mast cell degranulation by 15 minutes post-injection (Fig. 4.11(b), (d), (f)). However, a temporal dissociation was noted between mast cell degranulation and the time of highest neutrophil/eosinophil influx, this being 180 minutes post-injection for sP, and 360 minutes post-injection for both 48/80 and A23187 (Fig. 4.7 and Fig. 4.11). Speculatively, differential mediator release by mast cell activation using these different secretagogues could result in alterations in the rate of activation for neutrophils and eosinophils, these cells being more rapidly recruited as a consequence of sP-induced mast cell activation, in comparison to 48/80 and A23187.

The experiments discussed in this chapter have demonstrated that intradermal injection of sP and A23187, but not 48/80, produces a weal response in ovine skin. Dermal neutrophil and eosinophil influx (the latter not being evoked by sP), is accompanied by mast cell degranulation that occurs at some point before, or during, this cellular influx. There is thus sufficient evidence from this study to consider sP, 48/80 and A23187 as putative ovine cutaneous mast cell secretagogues. Assay for mast cell mediators in afferent lymph immediately after intradermal challenge with these agents would be a logical progression to prove that these agents are indeed true ovine cutaneous mast cell secretagogues. The use of these agents in prefemoral afferent lymphatic cannulation preparations would also allow the direct effect of mast cell activation on changes in dermal inflammatory cell trafficking to be determined (see Final Discussion).

Having defined putative secretagogue activity of sP, 48/80 and A23187 on the dermal mast cell population, it was of interest to further investigate the secretagogue effects of these agents on other ovine mast cell populations. This therefore led directly to the experiments described in Chapters 6 and 7, where optimal conditions for the development of recombinant ovine interleukin-3-dependent ovine bone marrow-derived mast cells (rOv.IL-3-dependent BMMC) were identified (Chapter 6), and where the secretagogue activity of these rOv.IL-3-dependent BMMC was compared to that observed in skin *in vivo*, using the same panel of putative secretagogues (Chapter 7).

CHAPTER 5

OVINE CUTANEOUS RESPONSES TO SHEEP MAST CELL PROTEINASE (SMCP).

5.1. Introduction : Chymases And Cutaneous Inflammation

Activation of skin mast cells results in the release of preformed granule mediators such as chymase, tryptase and histamine into the dermis (Caughey et al 1988a, Klein, Lavker and Murphy 1989), chymase being released in combination with proteoglycans (Schwartz et al 1981a, Sommerhoff, Ruoss and Caughey 1992). Indeed, after secretagogue-induced dermal mast cell activation, extracellular chymase has been demonstrated immunohistochemically in human dermis (Kaminer et al 1991). Released chymases and tryptases are believed to play a role in the initiation and modulation of cutaneous inflammatory responses (Harvima et al 1994, Harvima et al 1995, Schechter 1995), including the regulation of cutaneous neurogenic inflammation by proteolytic degradation of neuropeptides (Caughey et al 1988d, Walls et al 1992, Foreman 1993, Tam 1995).

The possible role of mast cell proteinases in the production of oedema has been considered for some time (Lagunoff and Benditt 1963). More specifically, chymases have been shown to affect the vascular response in inflammation (Seppa 1980), and to potentiate histamine-induced weal formation in canine skin (Rubenstein et al 1990).

The injection of a rat chymase in human skin evoked pruritus (Hagermark, Rajka and Berqvist 1972), and human mast cell chymase has been shown to convert keratinocyte-derived pro-interleukin-1β (Mizutani, Black and Kupper 1991) to active interleukin-1 (Mizutani et al 1991). This proinflammatory cytokine is part of an "axis" in the production of cutaneous inflammation (Kupper and Groves 1995), and can activate vascular endothelial cells as part of the inflammatory response (Pober 1988). When injected, human mast cell proteinases can evoke leukocyte emigration in skin (Fräki 1977), intradermal injection of human chymase evoking neutrophil infiltration in guinea pig skin (Walls et al 1994). Chymases can also alter the intercelluar matrix and basement membrane in skin (Sage, Woodbury and Bornstein 1979,

Seppa, Vaananen and Korhonen 1979, Briggaman et al 1984, Schechter 1989), to the extent that mast cells have been implicated in the pathogenesis of bullous pemphigoid (Goldstein, Wasserman and Wintroub 1989). Chymases have also been postulated to play a role in the removal of damaged tissue, thereby aiding in tissue repair (Lewis and Austen 1981). Chymases can also activate mast cells (Schick, Austen and Schwartz 1984, Schick and Austen 1986), thereby providing a possible mechanism for up-regulation of mast cell responses.

Thus, there is ample evidence in the literature to support the hypothesis that chymases (directly or indirectly) play a role in the initiation and modulation of cutaneous inflammatory responses. In support of its activity as a chymase, SMCP has recently been shown to cleave the neuropeptides substance P and bradykinin at P_1 Phe residues (Pemberton, Huntley and Miller, *submitted*), which may implicate a role for SMCP in cutaneous neurogenic inflammation. Unexpectedly however, SMCP also demonstrated trypsin-like activity against bovine and ovine serum albumin (Pemberton, Huntley and Miller, *submitted*). The tryptic activity of SMCP is defined by the cleavage of bonds in bovine albumin with a lysine at P_1 and aspartate or glutamate (acidic residues) at P_4 and P_4', a structure that occurs frequently in ovine albumin (Pemberton, Huntley and Miller, *submitted*). Although SMCP is present in only a small proportion of the ovine dermal mast cell population (Chapter 3), activation of cutaneous mast cells would be likely to release SMCP into the dermis, albeit at low concentrations. The availability of the technique of prefemoral afferent lymphatic cannulation would also allow, in later studies, dissection of any observed response to the intradermal injection of SMCP. Relatively few studies have investigated the effect of intradermally injected native mast cell proteinases on the cutaneous response in the homologous host *in vivo* (e.g. Fräki 1977, Rubenstein et al 1990). Intradermal injection of SMCP into the sheep therefore provided a suitable homologous system to investigate the *in vivo* function of a mast

cell granule chymase. The overall aim of the experiments described in this chapter was therefore to show if SMCP could evoke a cutaneous response in the homologous host *in vivo* and, if so, to define the nature of this response.

5.2. Experiment 5.1 - Cutaneous Responses To SMCP In The Sheep

5.2.1. Experimental Aim And Design

The aim of this study was to investigate the dose of SMCP which might evoke a cutaneous response, the kinetics of the response and the nature of the vascular and cellular reaction. The cutaneous response was assessed by measurement of weal volume and by quantifying the cutaneous mast cell and inflammatory cell changes histologically.

Four Blackface and four Finn-Dorset cross ewes were used. These had been grazed at pasture and had not had any anthelmintic treatment immediately before investigation. The group had been treated once with a topical ectoparasiticide (Deltamethrin 1% $^{w}/_{v}$, Coopers Spot On®) to remove lice and other ectoparasites four weeks before being housed, and once while housed.

The study was divided into two stages. In the first stage a dose response study was undertaken to assess whether SMCP could evoke a weal response in ovine skin and, if so, to determine the optimal dose. Histological examination of skin biopsies was undertaken for evidence of cellular infiltration and mast cell degranulation. In the second stage two separate time course studies (0-6 hours and 0-72 hours) were performed using the optimal dose of SMCP (based on weal volume data from the first stage).

Intradermal skin testing, weal volume measurement and skin punch biopsy was carried out on flank skin as described in 2.5.2.

5.2.1.1. Dose Response

SMCP and the SMCP diluent control were prepared as described (2.5.1.2, including absence of endotoxin) and 36µg, 3.6µg, 360ng or 36ng SMCP (in an injection volume of 50µl) were

injected intradermally into the skin of the left flank of four Blackface sheep. A biopsy of untreated skin was taken from one of the six grid sites immediately before the start of the study (untreated control, T0). Four of the remaining five sites were injected with SMCP, the final site being used for the diluent control. Weal volume was measured at 5, 10, 20, 60, 180 and 360 minutes after injection and biopsies were taken at 360 minutes.

5.2.1.2. Time Course - 0 to 6 Hours

This study was performed on the right flank of four Blackface sheep. A biopsy of untreated skin was taken from one of the six grid sites immediately before the start of the study (untreated control, T0). Four of the remaining five sites were injected intradermally with the optimal dose of SMCP, the final site being used for the diluent control. Weal volume was measured at 5, 10, 15, 60, 180 and 360 minutes after injection. Skin biopsies were removed 15, 60, 180 and 360 minutes after injection of SMCP. The diluent control was biopsied at the peak of the SMCP weal response, as determined from the dose response study.

5.2.1.3. Time Course - 0 to 72 Hours

This study was performed on the right flank of four Finn-Dorset cross ewes. The six grid sites on the flank were randomised, three sites being injected with the optimal dose of SMCP and three sites with the diluent control. Weal volume was measured at all sites at 15, 60, 180 and 360 minutes after injection to confirm that a characteristic immediate response was evoked. At 24, 48 and 72 hours the skin thickness was measured, and any increase from time zero noted. Skin biopsies from paired SMCP-treated and diluent control sites were taken at 24, 48 and 72 hours.

The weal volume (0 to 360 minutes) or change in skin thickness (24 to 72 hours) following injection of SMCP were compared with the appropriate controls by the Mann-Whitney U test (2.5.7). The number of mast cells, neutrophils, eosinophils (2.5.3) and the percentage of

extensively degranulated mast cells (2.5.4) in SMCP and diluent injected sites were compared by the Mann-Whitney U test (2.5.7).

5.2.2. Results

5.2.2.1. Weal Responses

A dose-dependent increase in weal volume was observed at 180 minutes after injection (Fig. 5.1(a)); significant increases in weal volume occurring with both 36µg and 3.6µg SMCP ($P<0.05$; Fig. 5.1(a)). The weal was circular to oval (no pseudopodia were present) and firm (non-fluctuating), with variable erythema and no flare. The intradermal injection of 36µg SMCP consistently resulted in a very large and erythematous firm weal, so 3.6µg SMCP was chosen as the optimal dose for time course studies. The peak response occurred at 180 minutes (Fig. 5.1(b)) and increased weal volumes were noted at 10, 15, 60 and 180 minutes ($P<0.05$; Fig. 5.1(b) and Fig. 5.1(c)). (N.B. As the diluent control had been biopsied at 180 minutes no control weal volume measurement was available at 360 minutes [Fig. 5.1(c)]).

Weals were not detected at 24, 48 and 72 hours and skin thicknesses were similar in SMCP- and diluent control-treated sites (Fig. 5.2). (N.B. The medians and ranges (n=4) for the weal volumes at these sites at 180 minutes after injection were 132.6 mm^3 (65.4 mm^3 - 198.9 mm^3) for 3.6µg SMCP and 1.66 mm^3 (0 mm^3 - 28.3 mm^3) for the diluent control ($P<0.0001$)).

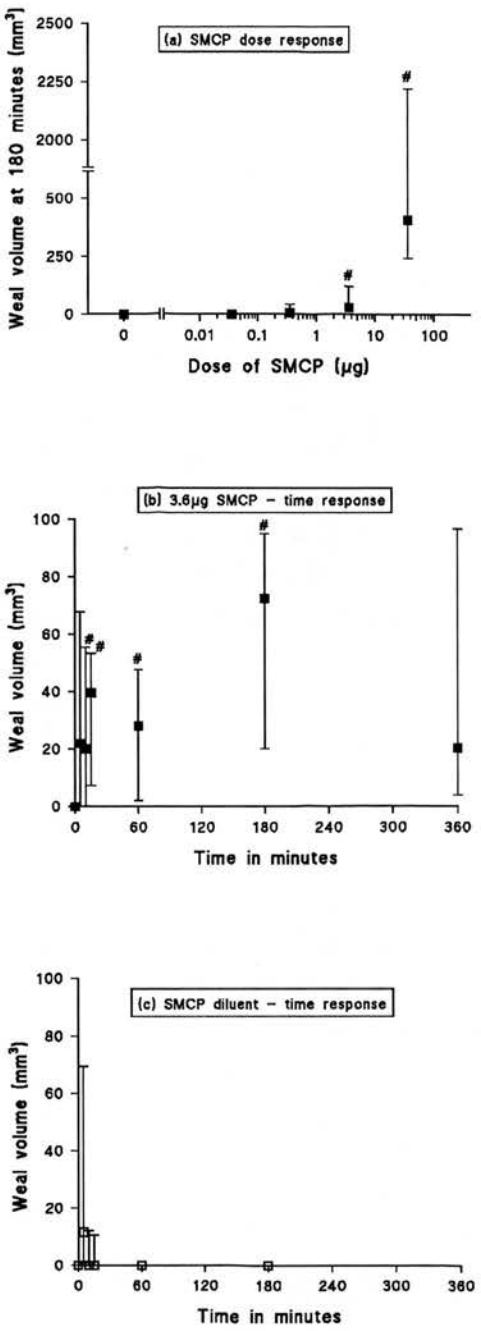


Figure 5.1. Weal volume following intradermal injection of **(a)** different doses of SMCP, measured 180 minutes later (this being the peak of the weal response to intradermal injection of SMCP assessed in time course studies, Fig. 5.1(b) and Fig. 5.1(c)); **(b)** 3.6µg SMCP and measured at varying intervals after injection and **(c)** diluent; measured until 180 minutes when the site was biopsied. (Median ± range; n=4). # $P<0.05$ compared with diluent.

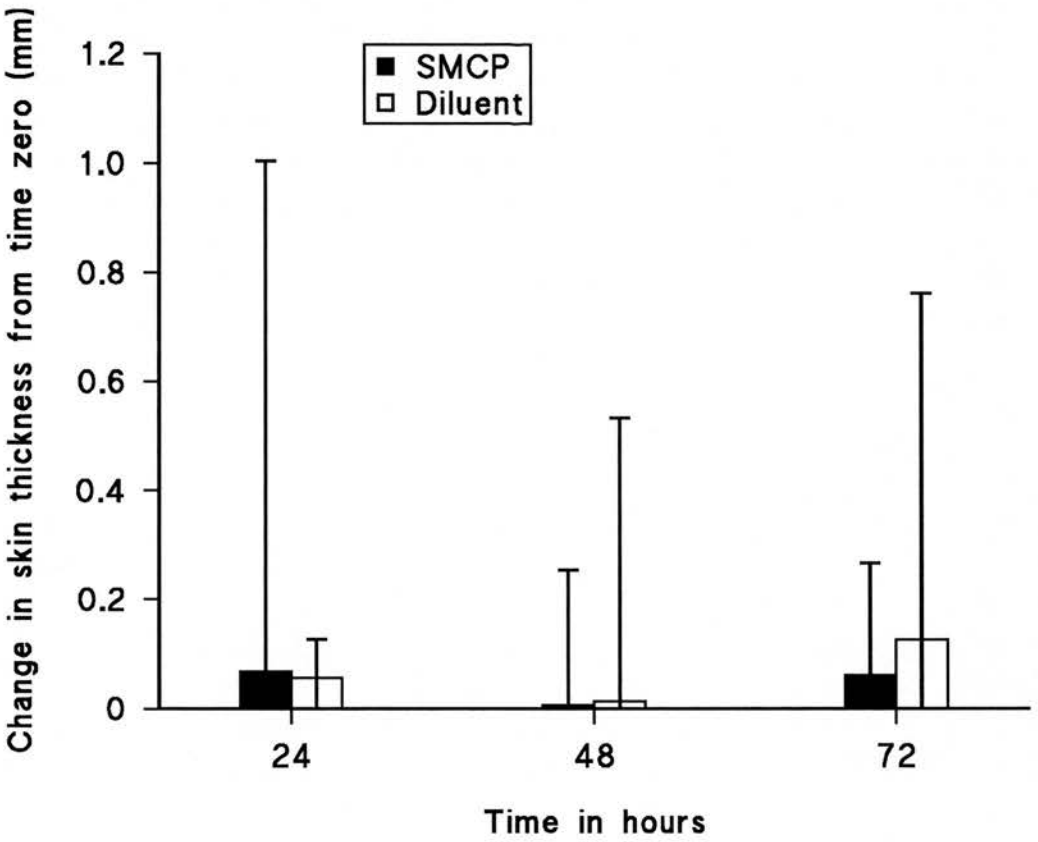


Figure 5.2. Change in skin thickness against time for sites treated with 3.6μg SMCP or the diluent control. (Median ± range; (n=4)).

5.2.2.2. Histology

5.2.2.2.1. Mast cells

The number of mast cells in the dermis remained unaltered regardless of the dose of SMCP (Fig. 5.3(a)) or time after injection (Fig. 5.3(b) and Fig. 5.4). However, the percentages of extensively degranulated mast cells (Fig. 5.5) in response to 36µg and 3.6µg SMCP ($P<0.05$; Fig. 5.6(a)) were increased 360 minutes after injection (e.g. for 3.6µg SMCP median 45.0%, range 37.5%-56.4%; for diluent median 20.9%, range 14.7% - 25.6%).

Increased degranulation also occurred 180 minutes after injection of 3.6µg SMCP ($P<0.05$; Fig. 5.6(b)). The extent of degranulation at the earlier time points was similar, indicating that degranulation probably occurred as early as 15 minutes after injection (Fig. 5.6(b)).

Background levels of degranulation (15%-25%) at time zero were similar to those obtained in diluent-treated sites at both 180 and 360 minutes after injection (Fig. 5.6(a) and Fig. 5.6(b)).

5.2.2.2.2. Neutrophils

SMCP evoked a dose-dependent influx of neutrophils into the dermis with significant increases being noted with 36µg, 3.6µg and 360ng SMCP ($P<0.05$; Fig. 5.7(a)). The neutrophil count increased gradually and was significant by 180 minutes after injection of 3.6µg SMCP when compared with diluent control ($P<0.05$; Fig. 5.7(b)). Counts continued to rise up to 360 minutes after injection (Fig. 5.7(b)), subsequently falling gradually over the period from 24 to 72 hours (Fig. 5.8). The neutrophil count in SMCP-treated sites declined markedly between 24 and 48 hours after injection ($P<0.05$; Fig. 5.8), although there were no significant differences when comparing SMCP-treated and diluent-treated sites at 24, 48 or 72 hours (Fig. 5.8).

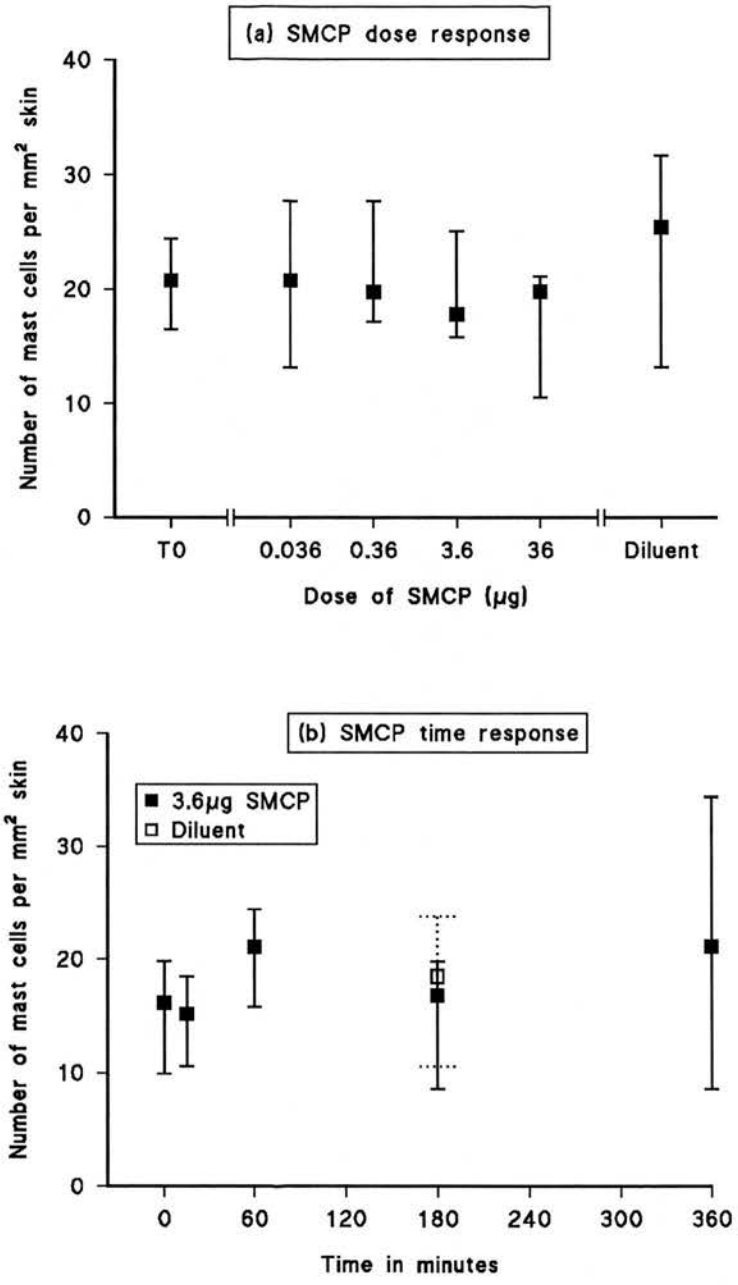


Figure 5.3. Mast cell counts in toluidine blue-stained sections following intradermal injection of **(a)** diluent and varying doses of SMCP measured 360 minutes later (T0 = zero time point); **(b)** 3.6µg SMCP and measured at varying intervals after injection, the diluent being measured at 180 minutes when the site was biopsied. (Median ± range; n=4).

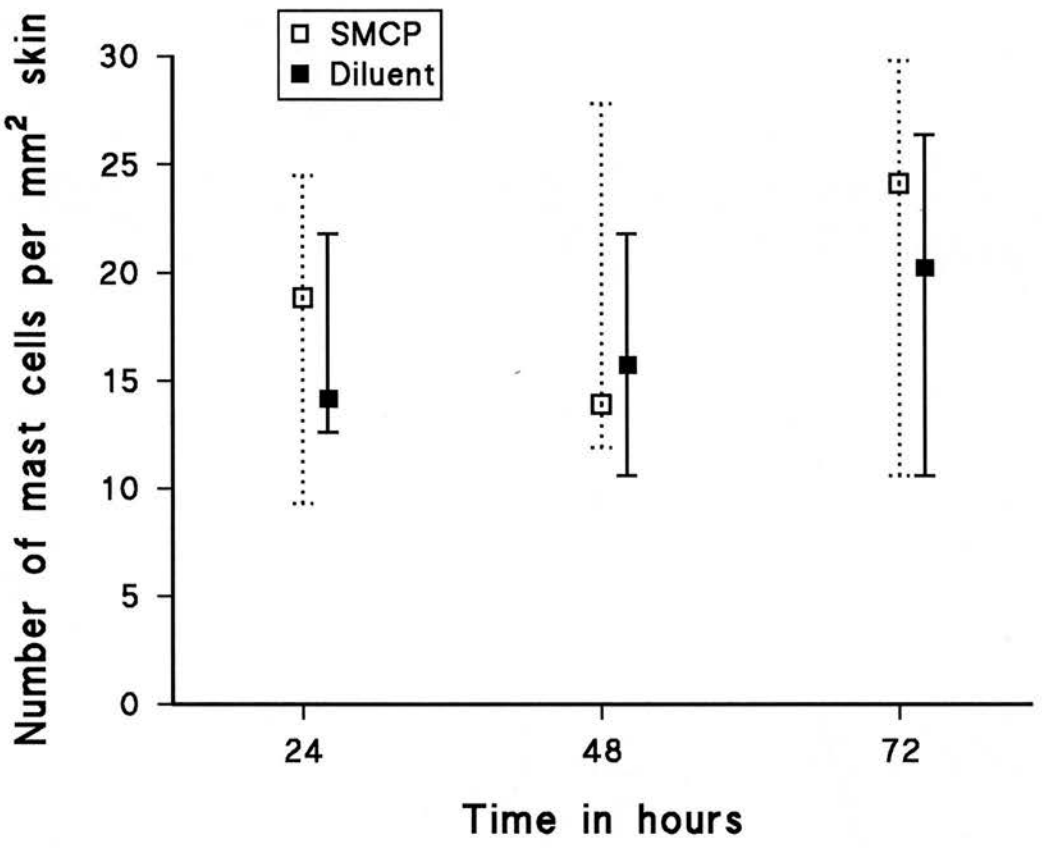


Figure 5.4. Mast cell counts in toluidine blue stained-sections following intradermal injection of 3.6µg SMCP and diluent assessed at 24, 48 and 72 hours after injection.
(Median ± range; n=4).

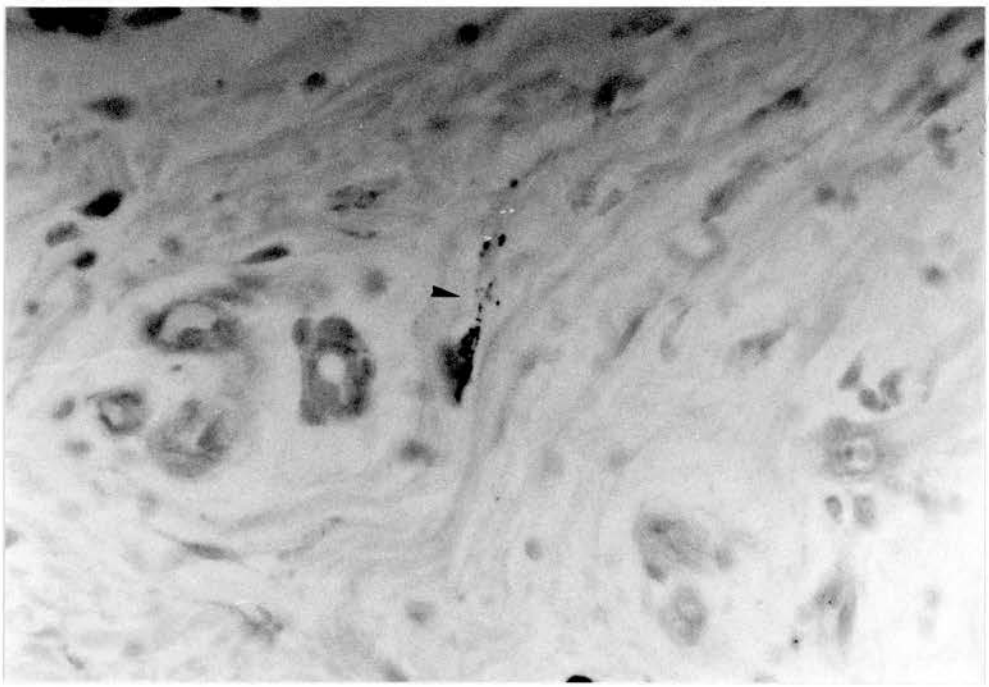


Figure 5.5. Degranulation of dermal mast cells at 180 minutes following injection of 3.6μg SMCP. Mast cell granules (arrowed) can be seen free in the dermis (toluidine blue x500).

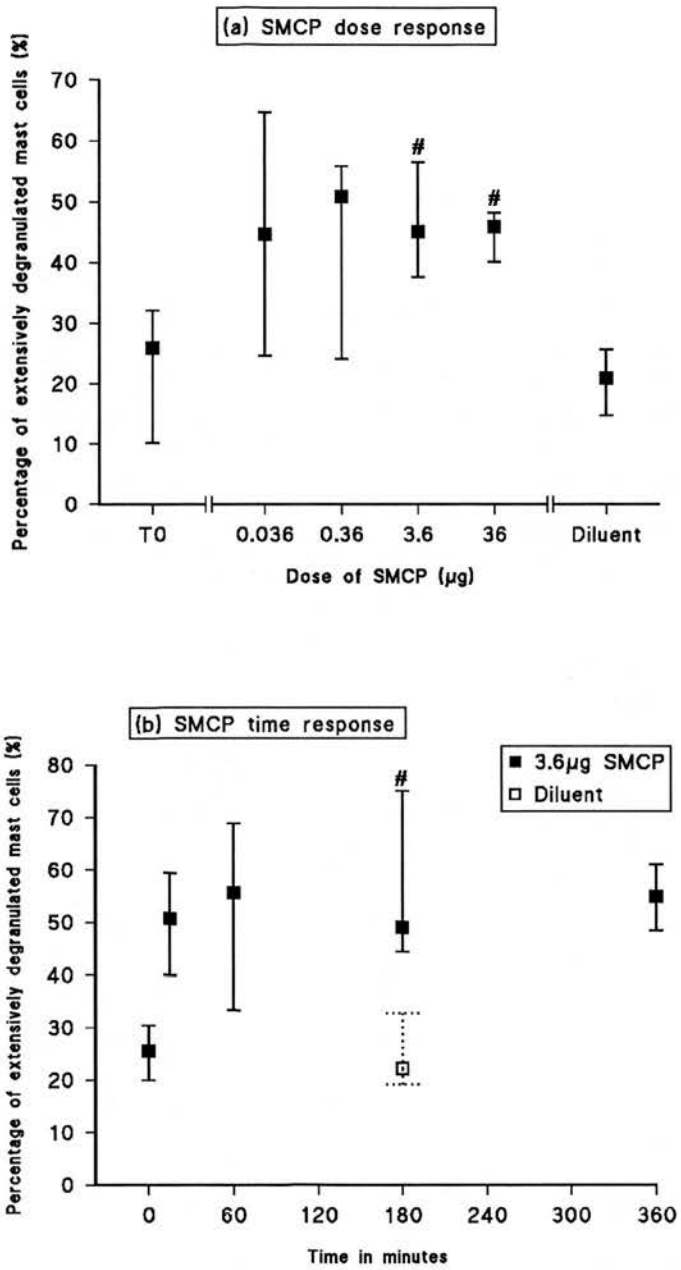


Figure 5.6. The percentage of extensively degranulated mast cells in toluidine blue-stained sections following intradermal injection of **(a)** diluent and varying doses of SMCP measured 360 minutes later (T0 = zero time point); **(b)** 3.6µg SMCP and measured at varying intervals after injection, the diluent being measured at 180 minutes when the site was biopsied. (Median ± range; n=4). # $P<0.05$ compared with the diluent control.

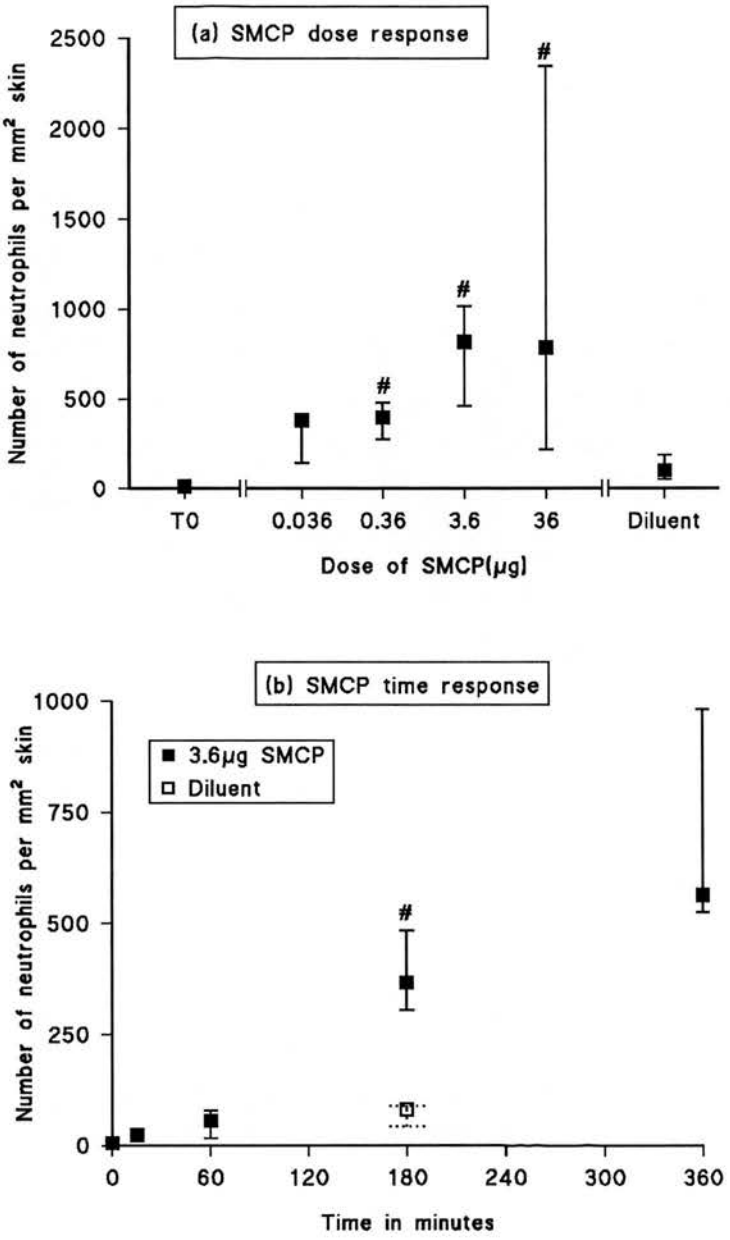


Figure 5.7. Neutrophil counts in Haematoxylin and Eosin-stained sections following intradermal injection of **(a)** diluent and varying doses of SMCP measured 360 minutes later (T0 = zero time point); **(b)** 3.6μg SMCP and measured at varying intervals after injection, the response to diluent being measured at 180 minutes when the site was biopsied. (Median ± range; n=4). # $P<0.05$ compared with diluent.

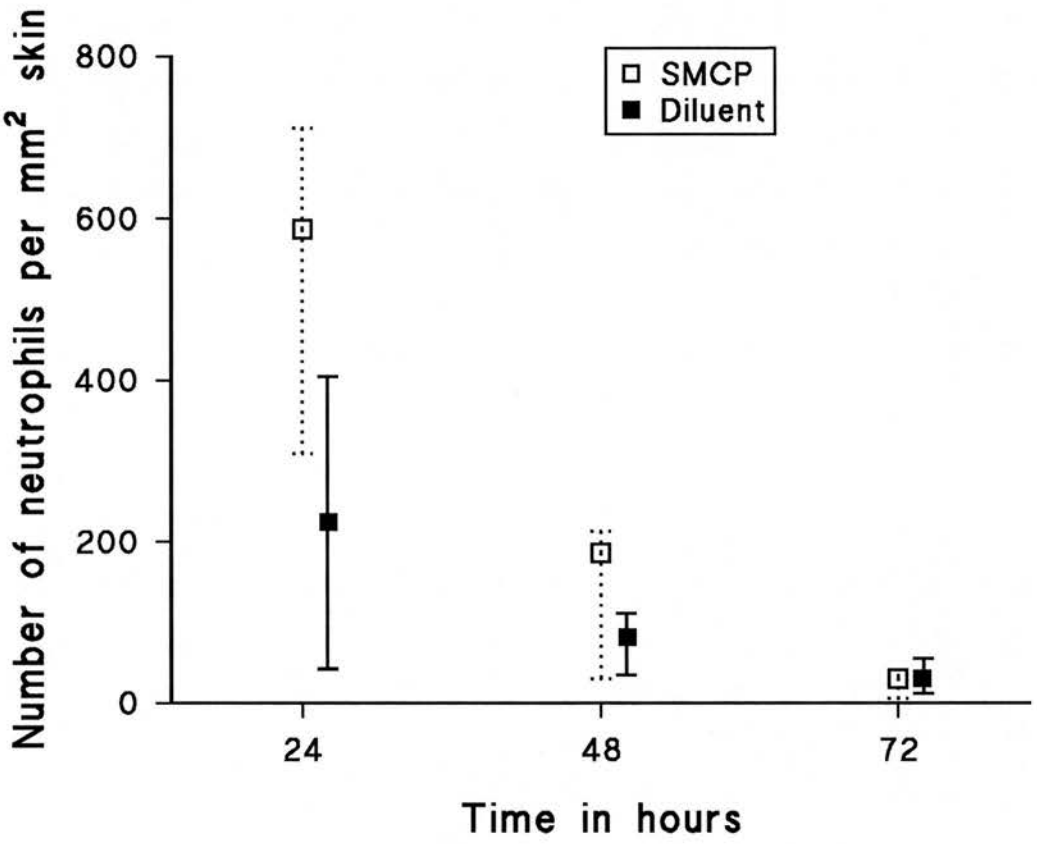


Figure 5.8. Neutrophil counts in Haematoxylin and Eosin-stained sections following intradermal injection of 3.6µg SMCP and diluent assessed at 24, 48 and 72 hours after injection. (Median ± range; n=4).

Congestion of dermal vessels was present in all animals 15 minutes after injection of 3.6μg SMCP (Fig. 5.9). Margination and emigration of neutrophils were apparent, predominantly in deep dermal vessels, at 60 minutes (Fig. 5.10) and there was an intense dermal neutrophilic infiltrate in sites biopsied at 180 ($P<0.05$; Fig. 5.7(b)) and 360 ($P<0.05$; Fig. 5.7(a)) minutes (Fig. 5.11).

5.2.2.2.3. Eosinophils

The number of eosinophils was increased at 360 minutes after injection of 36μg SMCP ($P<0.05$; Fig. 5.12(a)), but eosinophil counts were not increased at any time after injection of 3.6μg SMCP (Fig. 5.12(b) and Fig. 5.13).

The number of neutrophils far exceeded the number of eosinophils in skin sections for any given dose of SMCP (Fig. 5.7(a), Fig. 5.12(a)).

5.2.2.2.4. General histology from 0 to 6 hours after injection of SMCP

Marked dermal oedema with separation of dermal collagen fibres was noted in SMCP-treated sections, this being a dose-dependent effect (Fig. 5.14). Prominent bullous lesions were present at 360 minutes in two biopsies, with dermal-epidermal separation occurring at the level of the basement membrane (Fig. 5.15). (N.B. No bullous lesions had been noted in any skin section from any of the other studies described in this thesis).

5.2.2.2.5. General histology from 24 to 72 hours after injection of 3.6μg SMCP

No histological evidence of a major mononuclear cell infiltrate (lymphocytes or monocyte/macrophages) could be found in SMCP or diluent-treated sections at any of the time points examined.

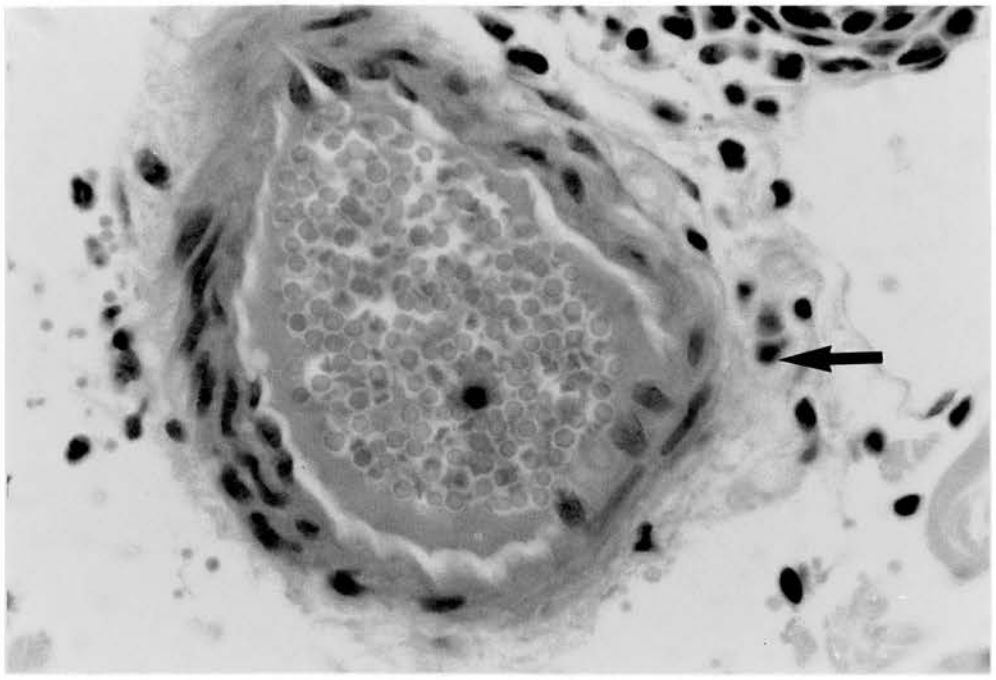


Figure 5.9. Congestion of dermal vessels (arrowed) in response to 3.6µg SMCP 15 minutes after injection. (H&E x500).

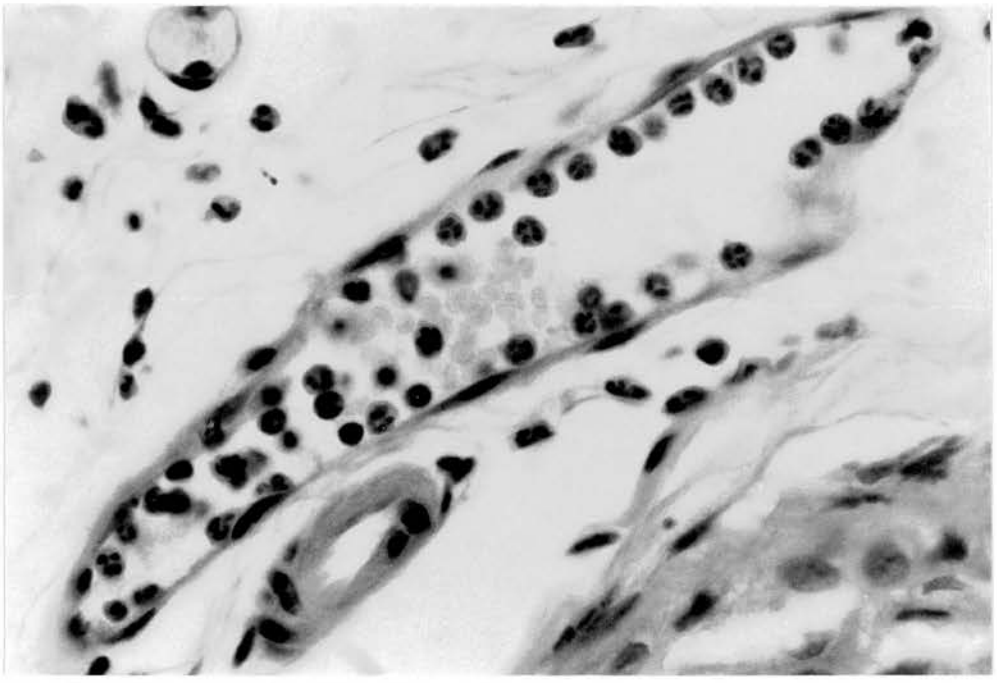


Figure 5.10. Profound margination and emigration of neutrophils in dermal vessels in response to 3.6µg SMCP at 60 minutes. (H&E x500).

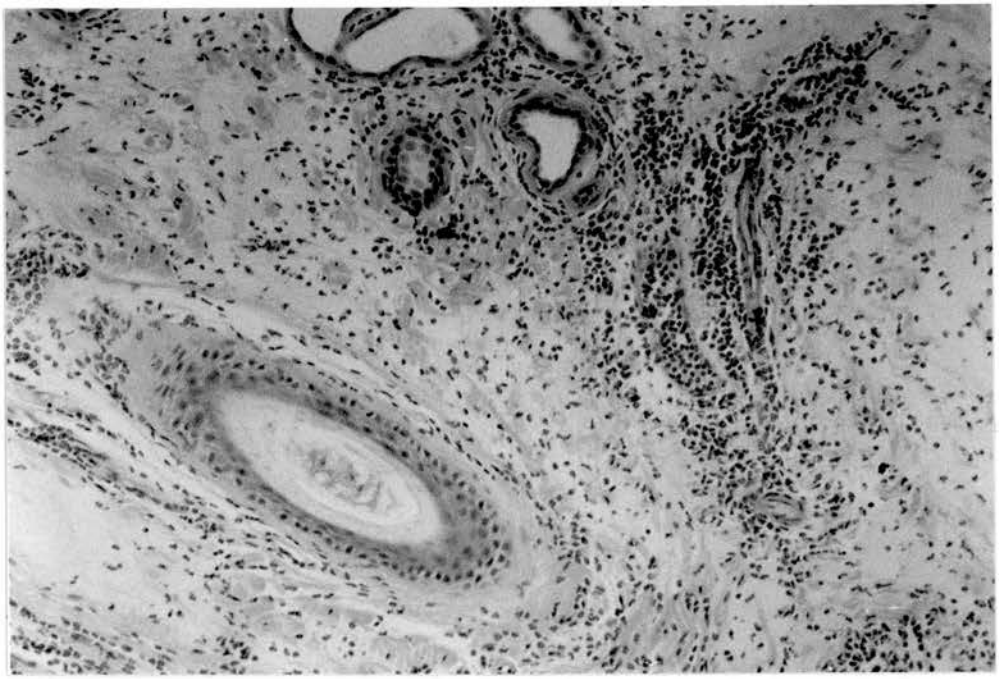


Figure 5.11. Intense dermal neutrophilic infiltrate in response to 3.6µg SMCP at 360 minutes. (H&E x125).

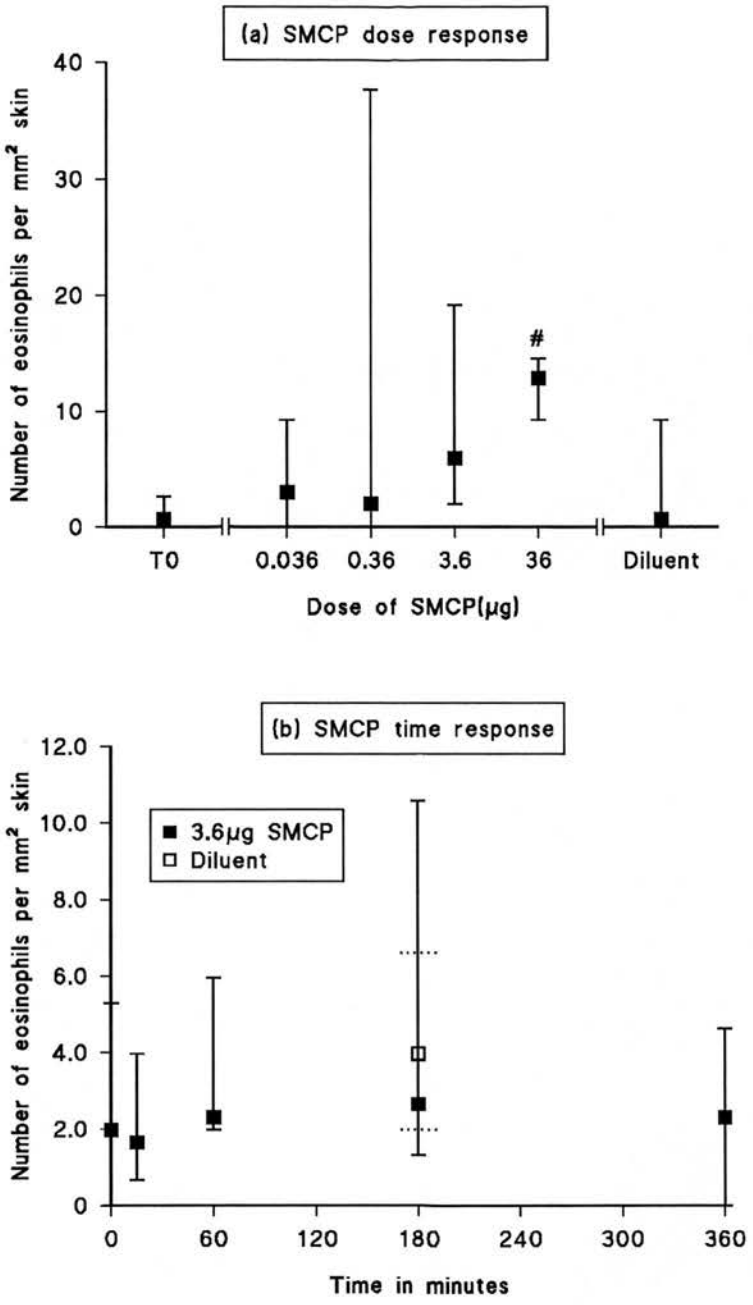


Figure 5.12. Eosinophil counts in carbol chromotrope-stained sections following intradermal injection of **(a)** diluent and varying doses of SMCP measured 360 minutes later (T0 = zero time point); **(b)** 3.6µg SMCP and measured at varying intervals after injection, the diluent being measured at 180 minutes when the site was biopsied. (Median ± range; n=4). # $P<0.05$ compared with diluent.

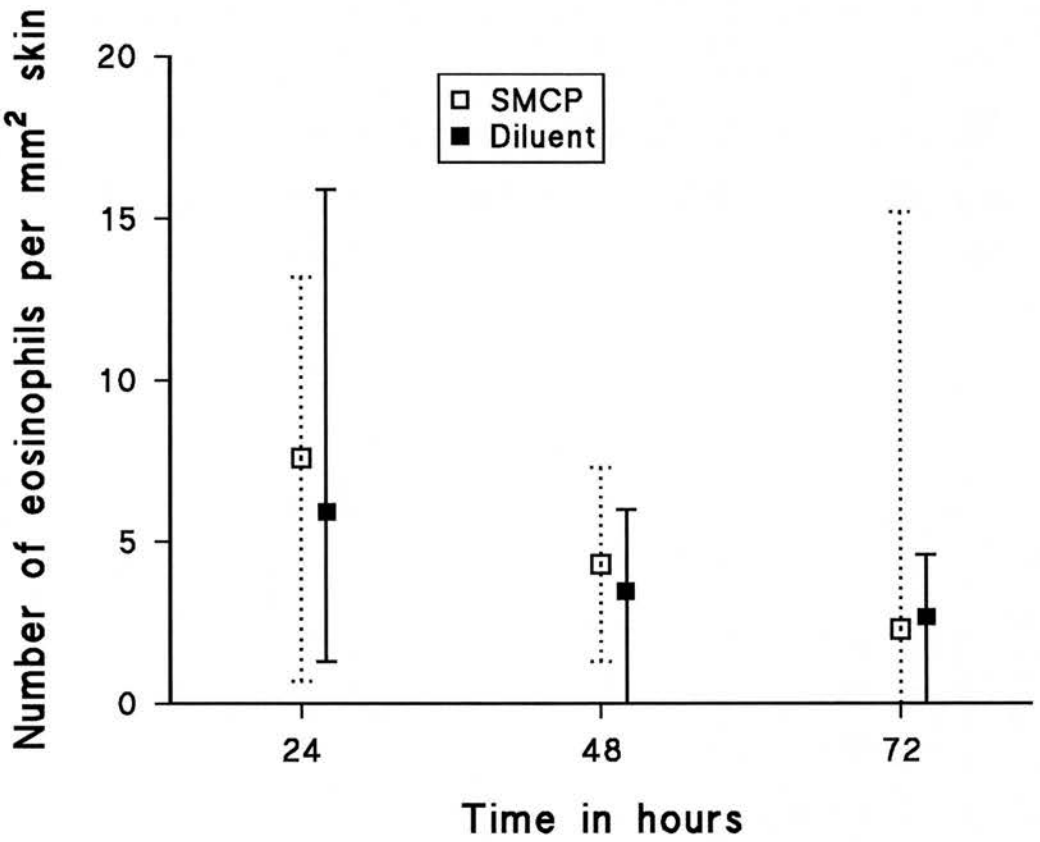


Figure 5.13. Eosinophil counts in carbol chromotrope-stained sections following intradermal injection of 3.6µg SMCP and diluent assessed at 24, 48 and 72 hours after injection. (Median ± range; n=4).

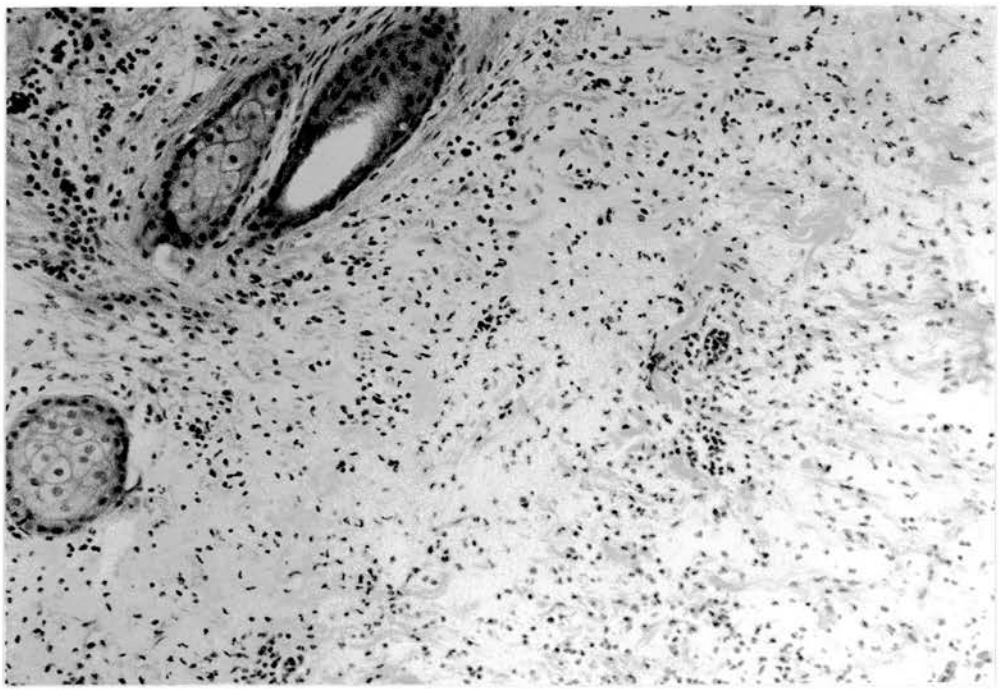


Figure 5.14. Marked dermal oedema evoked by 36µg SMCP at 360 minutes. (H&E x125).

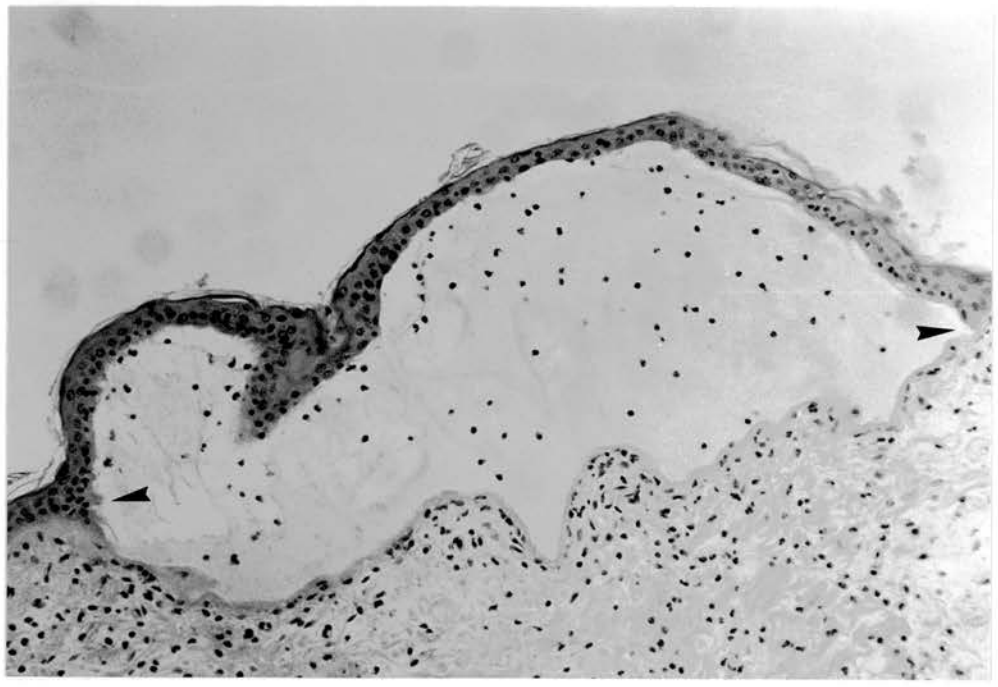


Figure 5.15. Bullous lesion in skin treated with 3.6µg SMCP at 360 minutes after injection. Note separation (arrowed) along the dermal-epidermal junction (H&E x125).

The results of these studies demonstrated that SMCP could evoke an immediate, but apparently not a delayed, response in ovine skin *in vivo*. The immediate response was characterized by production of a weal and marked neutrophil influx, with concomitant mast cell degranulation. A significant influx of eosinophils was noted only with the highest does of SMCP used (36μg), but this dose also resulted in an exaggerated weal response. Given that intradermal injection of 3.6μg SMCP resulted in a statistically significant weal response between 10 and 180 minutes after injection ($P<0.05$; Fig. 5.1(b) and Fig. 5.1(c)), and also resulted in both significant neutrophil influx ($P<0.05$; Fig. 5.7(a) and Fig. 5.7(b)) and accompanying extensive mast cell degranulation ($P<0.05$; Fig. 5.6(a) and Fig. 5.6(b)), this dose was chosen as optimal for use in further investigations.

5.3. Experiment 5.2 - The Cutaneous Response To Heat-Inactivated SMCP (HI-SMCP) In The Sheep

5.3.1. Experimental Aim and Design

The intradermal injection of SMCP was shown to evoke an immediate cutaneous response (Experiment 5.1). The aim of this study was to investigate whether this occurs as a consequence of the proteolytic activity of SMCP, or whether it results as a consequence of the inherent property of SMCP to activate cells as a basic, polycationic protein.

Eight Finn-Dorset cross ewes were used, maintained and housed as previously described (Experiment 5.1). SMCP and the diluent control were prepared (2.5.1.2), aliquots of each being subjected to heat inactivation (HI) at 64°C for 10 minutes (2.5.1.3), this resulting in SMCP with a residual activity of approximately 2% as described (2.5.1.3). Aliquots of SMCP (3.6μg) and of HI-SMCP and of diluent and HI-diluent were prepared for intradermal injection. The six grid sites on the skin of the left flank were randomised, two sites being left untreated. The four remaining sites were injected with SMCP, HI-SMCP, diluent or HI-diluent. Weal volume was measured at 0, 15, 60, 180 and 360 minutes after injection and biopsies were taken at 360 minutes.

Weal volumes were compared by the Mann-Whitney U test (2.5.7). The number of mast cells and neutrophils (2.5.3), and the percentage of extensively degranulated mast cells (2.5.4) in skin sections were compared by the Mann-Whitney U test (2.5.7).

5.3.2. Results

5.3.2.1. Weal Responses

Heat inactivation of SMCP abrogated the immediate weal response (Fig. 5.16(a)). This effect was significant at all time points examined ($P<0.05$-$P<0.01$; Fig. 5.16(a)).

(As anticipated from previous studies, SMCP evoked a significant weal response in comparison to diluent at 15 and 60 minutes after injection (Fig. 5.16(a) and Fig. 5.16(b)). There was no difference in weal volume between HI-SMCP and HI-diluent-treated sites at these time points (Fig. 5.16(a) and Fig. 5.16(b)).

5.3.2.2. Histology

5.3.2.2.1. Mast cells

There was no effect of heat inactivation of SMCP or diluent on the number of mast cells (Fig. 5.17(a)). However, the percentage of extensively degranulated mast cells in SMCP-treated in comparison to diluent-treated sites was increased ($P<0.05$; Fig. 5.17(b)). Despite the range of values for SMCP and HI-SMCP overlapping, there was no significant effect when comparing HI-SMCP against HI-diluent. Again, similar to results in Experiment 5.1, there was a relatively high background of extensive degranulation (30%-35%) in both diluent and HI-diluent-treated sites (Fig. 5.17(b)).

5.3.2.2.2. Neutrophils

An increased dermal neutrophil count was noted when comparing both values for SMCP against diluent, and for HI-SMCP against HI-diluent ($P<0.05$; Fig. 5.18). There was no effect of heat inactivation of SMCP on the degree of neutrophil influx (Fig. 5.18). Therefore, in contrast to the marked effect on the weal response (Fig. 5.16(a)), heat inactivation of SMCP failed to abrogate the observed neutrophil influx.

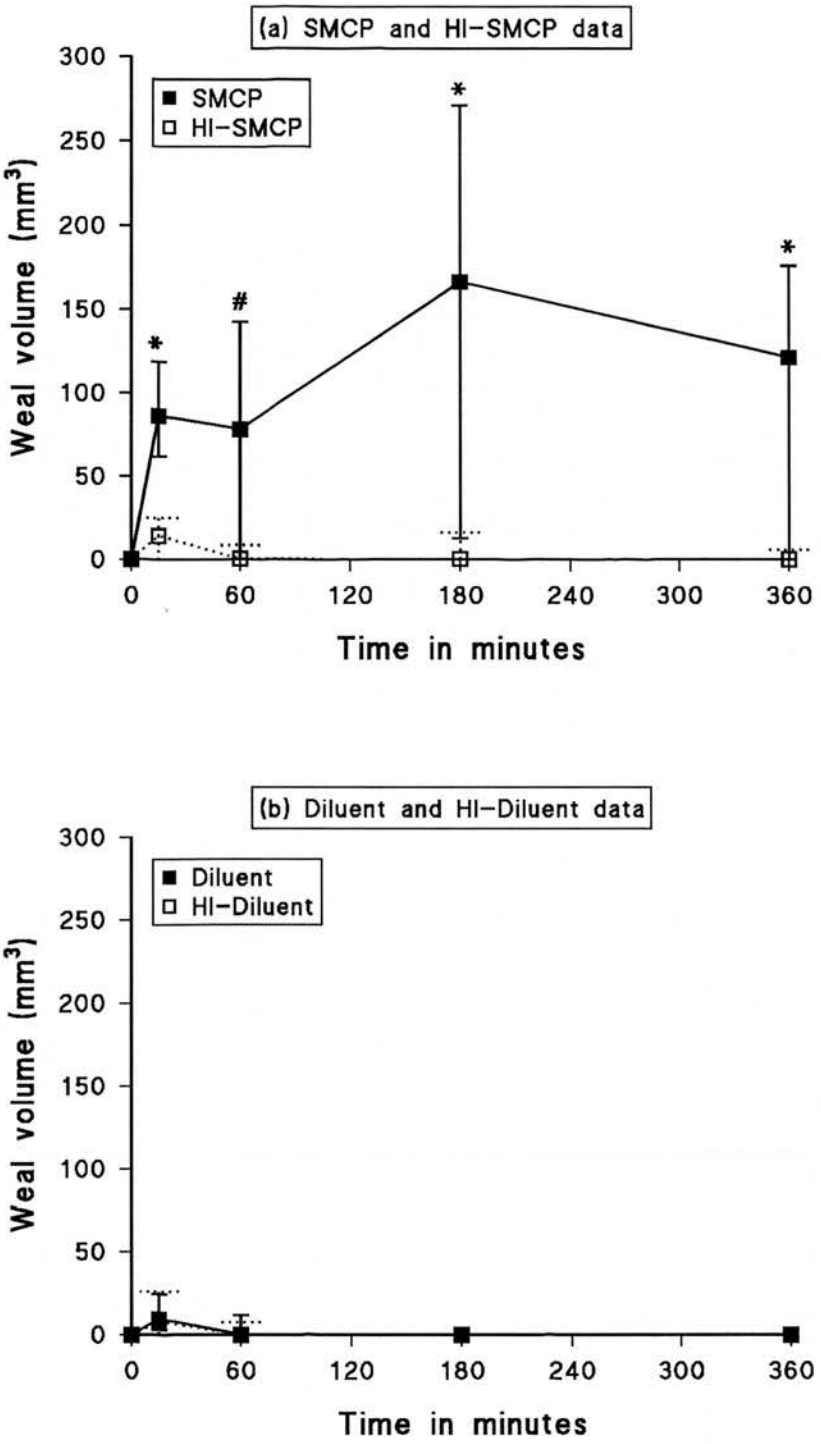


Figure 5.16. Weal responses evoked by intradermal injections of **(a)** SMCP (3.6µg) or HI-SMCP, and **(b)** diluent or HI-diluent. (Median ± range; n=8). # $P<0.05$ compared to HI-SMCP and * represents a similar effect at $P<0.01$.

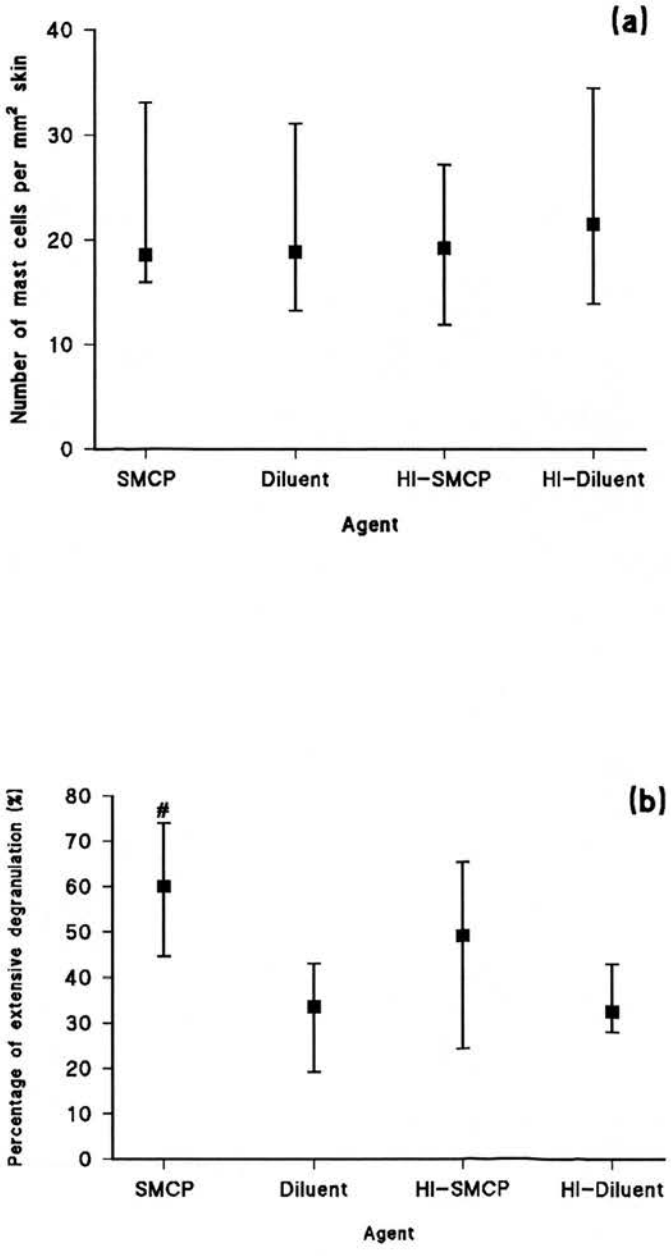


Figure 5.17. The effect of heat inactivation of SMCP (3.6μg) on **(a)** the number of mast cells and **(b)** the percentage of extensively degranulated mast cells in toluidine blue-stained sections. (Median ± range; n=8). # $P<0.05$ compared to diluent.

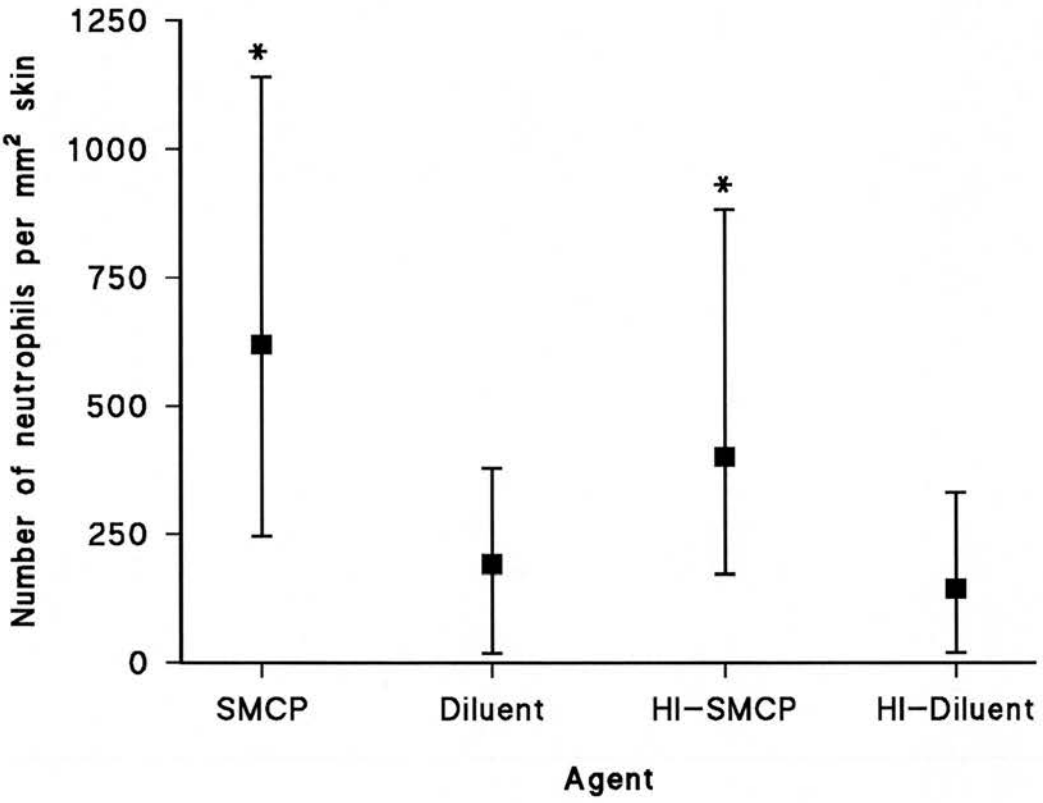


Figure 5.18. The effect of heat inactivation of SMCP (3.6μg) on neutrophil influx in Haematoxylin and Eosin-stained sections. (Median ± range; n=8). * $P<0.05$ when comparing SMCP or HI-SMCP against the respective diluent or HI-diluent.

5.4. Discussion

The finding that SMCP evokes both a weal response and neutrophil infiltration in skin is consistent with results from other studies investigating the activity of skin proteinases and chymases (Fräki 1977, Walls et al 1994), the present study being particularly significant in that SMCP is used in the homologous host. It is interesting that heat inactivation of SMCP abrogated the weal response, but had little effect on the extent of neutrophil influx. The heat-inactivated SMCP had a residual activity (2.5.1.3) of about 2%. Thus, it can be deduced that the injection of 3.6µg heat-inactivated SMCP would be equivalent to injecting 72ng of active SMCP. From the dose response study, it can be seen that neutrophil influx (Fig. 5.7(a)), without a weal response (Fig. 5.1(a)), could be evoked by such a dose. The same dose of SMCP also tended to increase the percentage of extensively degranulated mast cells (Fig. 5.6(a)), approximating the result noted with inactivated SMCP in Experiment 5.2 (Fig. 5.17(b)). The ideal solution to this problem would be to identify an active-site inhibitor for SMCP, which would leave the SMCP molecule intact, yet leave the chymase enzymatically inactive. Subsequent repetition of Experiment 5.2 would determine if SMCP inactivation abrogates *both* the weal response and neutrophil influx. Lack of time precluded co-injection of SMCP with protease inhibitors to investigate this further, although soya-bean trypsin inhibitor (SBTI) would have been the most appropriate to use in terms of the degree of likely inhibition (Pemberton, Huntley and Miller, *submitted*). Although denaturing the protein would have achieved complete inactivation of the active site, there would have been concomitant changes to the tertiary structure of SMCP, which again may have affected results.

Although the investigations in this Chapter have defined the biological nature of the cutaneous response to SMCP, the underlying mechanisms remain open to speculation and are ripe for further study. The cutaneous response to SMCP clearly involves both a vascular response

(responsible for significant weal formation within 10 minutes of injection [Fig. 5.1(b)]) and a cellular response (with margination and subsequent emigration of neutrophils across dermal vascular endothelium increasing gradually from 0 to 6 hours after injection [Fig. 5.7(b)]), which may occur due to separate, or possibly inter-related, mechanisms. Mast cell degranulation accompanies the cutaneous response, this apparently occurring within 15 minutes of injection (Fig. 5.6(b)). This is consistent with *in vitro* studies on rat peritoneal mast cells, which can be directly activated by chymase (Schick, Austen and Schwartz 1984). To determine if SMCP could directly activate ovine mast cells, it was used subsequently in mediator release studies on a rOv.IL-3-dependent BMMC population generated *in vitro* (Chapter 7). If SMCP could, as with rat chymase, activate mast cells directly, preformed mediators (including histamine) would be released, and other mediators and cytokines synthesised (Gordon, Burd and Galli 1990). Histamine usually evokes an immediate but relatively transient vascular response with leakage from dermal venules giving rise to weal formation (Pasyk and Cherry 1990). In the dog, chymase was shown to potentiate the weal response induced by histamine release from dermal mast cells (Rubenstein et al 1990). In contrast to the dog, where the H_1 receptor antagonist pyrilamine completely inhibits the chymase-induced weal response (Rubenstein et al 1990), human chymase-induced responses in guinea pig skin are not inhibited by H_1 and H_2 antagonists (Walls et al 1993). SMCP could therefore act to potentiate the weal response induced by histamine release from ovine dermal mast cells. However, the effect of H_1 antagonists on the SMCP-induced response could not be investigated, owing to lack of available time. The contribution of histamine to the SMCP-induced weal response could be classically deduced by the use of antihistamines (H_1 and H_2 antagonists) *in vivo*. However, as dermal mast cells in the rat have recently been shown to possess H_3 receptors (which may constitute a negative feedback mechanism for mast cell histamine release (Ohkubo et al 1994), a similar ovine mechanism, if present, may lead to

attenuation of histamine responses independently of the action of administered H_1 antagonists and therefore complicate interpretation of results.

Newly generated lipid-derived mediators may be involved in the cutaneous response to SMCP. Leukotriene (LT) C_4 and LTD_4 have been shown to be 1000 times more potent in promoting plasma leakage than histamine (Dahlen et al 1981), whereas LTB_4 evokes adhesion of leukocytes to endothelium in postcapillary venules (Dahlen et al 1981), this adhesion being mediated via non-cyclooxygenase-dependent mechanisms (Buchanan, Vazquez and Gimbrone 1983). Additionally, the phosphoglyceride platelet-activating factor is generated by human vascular endothelial cells in response to histamine or IL-1 (Camussi et al 1983) and has been shown to evoke increased vascular permeability and leukocyte influx in rabbit skin (Humphrey et al 1984). Thus, there is ample evidence that histamine and other mediators from activated mast cells or endothelium could play a role in the cutaneous response to SMCP.

Activated mast cells can also generate a wide range of pro-inflammatory cytokines (particularly IL-1 and TNF-α; Gordon, Burd and Galli 1990), which can in turn directly activate vascular endothelial cells to both generate cytokines and undergo functional and morphological changes that result in the margination and subsequent emigration of leukocytes (Cotran 1987). Certainly, in human skin, dermal mast cells contain and release TNF-α upon activation, this directly inducing ELAM-1 (endothelial cell adhesion molecule-1) expression in superficial dermal venules (Walsh et al 1991). ELAM-1, which is exclusively expressed by cytokine-activated endothelial cells, mediates adhesion of leukocytes to endothelial cells (Walsh, Lavker and Murphy 1990). Margination and emigration of neutrophils was a prominent feature of the cutaneous response. The kinetics of ELAM-1 expression following secretagogue-induced human dermal mast cell activation (maximal by 6 hours, declining by 24

hours and absent by 48 hours after activation; Klein et al 1989) are very similar to the kinetics of SMCP-induced neutrophil influx (Fig. 5.7(b) and Fig. 5.8), and may support the hypothesis that mast cell activation plays a role in the ovine cutaneous response. Interleukin 8 (IL-8), a "C-X-C" chemokine, can be produced from endothelial cells activated by primary cytokines such as the interleukins and TNF-α (Schröder 1995). IL-8 evokes marked neutrophil influx in human (Swensson et al 1991), rabbit (Colditz, Zwahlen and Baggiolini 1990) and sheep skin (Seow et al 1994). Also, inactive primary cytokine precursors could be cleaved by SMCP to produce pro-inflammatory cytokines that activate endothelium (chymase has been shown to cleave pro-IL-1β in human skin; Mizutani et al 1991), one effect of which may be to generate IL-8. Alternatively, vascular endothelium may be directly activated by SMCP via protease-activated receptors (Altieri 1995, Coughlin 1994), as has been described for both cathepsin G and thrombin (Peterson, Stone and Shasby 1987, Aschner et al 1990). In addition to the possible paracrine effect of cytokines on local vascular endothelium produced by neutrophils recruited to the site (Lloyd and Oppenheim 1992), it is believed that neutrophils can play a direct role in the control of vascular permeability in skin, thereby contributing to oedema (Issekutz 1981, Wedmore and Williams 1981). Theoretically therefore, a marked neutrophilic infiltrate can potentiate oedema and it is interesting to note that, in the case of SMCP, the greatest increase in median neutrophil influx (Fig. 5.7(b)) is coincident with the peak of the weal response.

Therefore, the mechanisms of the vascular and cellular responses to SMCP injection alluded to above are probably closely inter-related and likely to involve mast cell activation at some stage of the response, particularly as mast cells are believed to help regulate the second (chemoattractant) phase of leukocyte margination (Springer 1994).

It became apparent during the investigations that there was a relatively high background (20%-30%) of degranulated mast cells in untreated control biopsies. Similar values were obtained for diluent control sections (Fig. 5.6(a)). This implied that the diluent itself was not evoking the degranulation, but that it could be due either to the mechanical effect of biopsy removal or due to a fixation artefact (due to slow penetration of skin by fixative). Additionally, it was noted that these relatively high levels of mast cell degranulation in diluent control sites were not accompanied by proportionately high neutrophil counts (e.g. Fig. 5.6(a) and Fig. 5.7(a)), which would again support the hypothesis of a mechanical or fixation artefact.

The finding in two biopsies of apparent bulla formation is interesting, particularly as chymases have been shown to degrade the basement membrane in skin (Briggaman et al 1984) and may play a role in bullous pemphigoid (Goldstein, Wasserman and Wintroub 1989). However, this was not a consistent finding, and it must be remembered that similar lesions can be produced by both poor fixation and mechanical damage of skin biopsies (Scott, Miller and Griffin 1995). Further investigation would be required to confirm that the latter possibility was not the case.

Having shown that SMCP evokes a cutaneous response, its use in ovine afferent lymphatic preparations would allow direct evaluation of its affect on cutaneous mast cells (by assay for mast cell mediators in afferent lymph) and on the inflammatory response directly *in vivo*, thereby addressing some of the points raised above (see Final Discussion).

CHAPTER 6

THE GENERATION AND DEVELOPMENT OF RECOMBINANT OVINE INTERLEUKIN-3-DEPENDENT OVINE BONE MARROW-DERIVED MAST CELLS (rOv.IL-3 BMMC) *IN VITRO*.

6.1. Introduction

The generation of mast cell populations *in vitro* has helped elucidate mast cell heterogeneity in the rat, mouse and man. Rat BMMC are considered analogous to the MMC phenotype (Haig et al 1982, Miller 1993a). Unlike rat CTMC, both populations stain with alcian blue, are dependent upon T cell factors for development, apparently synthesize chondroitin sulphate diB rather than heparin as the major proteoglycan (Stevens et al 1986b) and produce leukotriene C_4 rather than prostaglandin D_2 as the major arachidonic acid metabolite upon activation. Both BMMC and MMC express RMCP II (Haig et al 1982, Gibson and Miller 1986) and not RMCP-I (M^{c}Menamin et al 1987), the latter being associated with the CTMC phenotype. Importantly, IL-3-dependent BMMC represent a homogeneous cell population with respect to proteinase expression (Haig et al 1988a).

Similarly, murine BMMC were originally considered as being analogous to the MMC phenotype. Both populations contained chondroitin sulphate E rather than heparin (Sredni et al 1983), synthesized LTB_4 or LTC_4 in preference to PGD_2 upon activation (Razin et al 1982) and could not be activated by compound 48/80 unlike murine CTMC (Katz, Stevens and Austen 1985). However, further studies revealed that murine BMMC are heterogeneous with respect to proteinase phenotype (Newlands et al 1991). Thus, although there a number of biochemical and functional similarities, BMMC cannot be considered the true analogue of MMC in the mouse.

In man, mast cells have been classified into two types, based on their content of tryptase alone (MC^T) or of tryptase and chymase (MC^{TC}) (Irani et al 1986). For example, MC^{TC} account for 99% of dispersed skin mast cells (Schwartz et al 1987), whereas MC^T predominate in intestinal mucosa (Irani et al 1986). Proteinase heterogeneity also exists in cultured human mast cells *in vitro* with cells cultured from foetal liver progenitors being largely MC^T (Irani et

al 1992), whereas those derived from cord blood mononuclear cells are predominantly MC^{TC} (Furitsu et al 1989).

In the sheep, mast cell heterogeneity *in vivo* has been defined on the basis of the distribution of SMCP (Chapter 3). Thus, in the gastrointestinal tract mast cells are overwhelmingly SMCP-positive (the putative MMC subset), whereas dermal mast cells are predominantly SMCP-negative (the putative CTMC subset). Ovine mast cells have been previously generated *in vitro* from bone marrow cells grown in the presence of lymphocyte-derived conditioned medium (CM) (Haig et al 1988b, Huntley et al 1992). These CM-BMMC were SMCP-positive, but apparently contained an additional serine proteinase detected by Western blotting that was absent in isolated abomasal MMC (Huntley et al 1992). Thus, proteinase heterogeneity may occur in ovine CM-BMMC, and it was hypothesized that CM-BMMC may represent a mixed population of MMC and CTMC. MMC and CM-BMMC both contained arylsulfatase, β-hexosaminidase, histamine and dopamine, indicating that there were similarities between the two cell types (Huntley et al 1992). However, as CM-BMMC were SMCP-positive, these could not be considered the *in vivo* analogue of the majority of ovine dermal mast cells. Thus, an alternative source of ovine BMMC was sought for use in future mediator release studies, enzymatic dispersion of ovine dermal mast cells having proved problematical (J.F.Huntley, *personal communication*).

When grown in the presence of IL-3, bone marrow cells from the mouse (Ghiara et al 1985, Rennick et al 1985, Chiu and Burrall 1990) and rat (Haig et al 1988a) differentiate into mast cells. However, in human bone marrow cultures supplemented with IL-3, the production of basophils is favoured (Kirshenbaum et al 1989, Valent et al 1989), although small numbers of mast cells can be generated (Kirshenbaum et al 1989, 1992). Following cloning and expression of the ovine IL-3 gene (M^{c}Innes, Haig and Logan 1993), it was known that

recombinant ovine IL-3 (rOv.IL-3) could support the development of mast cells from ovine bone marrow *in vitro* (Haig 1993), based on staining both with Leishman's stain and to detect the presence of chloroacetate esterase. However, studies to optimise the growth of rOv.IL-3 BMMC in a liquid culture system were not undertaken, and no attempt had been made to characterize the rOv.IL-3 BMMC with regard to their possible phenotypic heterogeneity.

The aims of the investigations described in this Chapter were therefore firstly to define the culture conditions required to generate rOv.IL-3-dependent BMMC *in vitro*, and secondly to characterize them in terms of their mediator content. These aims were achieved in three stages. (1) The optimal dose of rOv.IL-3 required to generate BMMC was determined in dose response studies. (2) The kinetics of BMMC development was defined in a time course study using the optimal dose of rOv.IL-3. (3) Finally, in an effort to increase the percentage of BMMC obtained in culture, the effect of transferring the non-adherent cell population at feeding to fresh wells was investigated.

6.2. Experiment 6.1 - The Generation And Development Of rOv.IL-3 BMMC *In Vitro*

General Experimental Design

Ovine bone marrow was obtained from a Blackface lamb (2.2), the purified cell population being plated out in 24-well plates as described (2.3.1). Both test (using 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} rOv.IL-3 in the dose response study and 10^{-1} rOv.IL-3 in the remaining experiments) and control (IMDM/10% FCS (Appendix A)) samples were plated out in triplicate wells.

At harvest, a cell count was performed for each well (2.3.3) and cytosmear preparations made (2.3.4) for both histochemical (Leishman's, 2.3.5) and immunohistochemical (to detect SMCP, 2.3.6, 2.3.8.2) staining. The percentage of mast cells in anti-SMCP stained

cytosmears could therefore be calculated (2.3.10). The remaining cells in each well were assayed as a cell pellet (2.3.2) for the presence of SMCP, and for arylsulfatase and β-hexosaminidase activity as described (2.3.11, 2.3.12, 2.3.13, 2.3.14). Results were expressed as the quantity or activity of mediator present in each well. Where statistical significance could be demonstrated between groups by ANOVA, Student's *t*-test was subsequently performed to compare test groups against the appropriate control. Additionally, in the time course study, linear regression analysis was used to correlate the cell mediator content (for SMCP) or activity (for arylsulfatase or β-hexosaminidase) against both the total number of cells and the absolute number of SMCP-positive mast cells for each well.

6.2.1. Dose Response Study

The aim was to determine the optimal dose of rOv.IL-3 required for mast cell development *in vitro*. Final dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} rOv.IL-3 (expressed as dilutions of the original CHO cell-derived supernatant) were used, these being added to cultured cells in three 24 well plates in conjunction with triplicate control wells (IMDM/10%FCS alone). Individual plates were harvested on days 6, 12 and 17 of culture (2.3.2).

6.2.1.1. Results

There was a dose-dependent increase in viable cell count in the presence of rOv.IL-3 on days 12 and 17 of culture in comparison to IMDM/10% FCS controls (For dilutions of 10^{-1}, 10^{-2} and 10^{-3} rOv.IL-3 on both days $P<0.05$-0.01; Fig. 6.1). This dose-dependent effect on cell density was reflected in Leishman's-stained cytosmears (Fig. 6.2). Increasing numbers of granulated, mononuclear cells were identified in cytosmears from wells cultured with higher concentrations of rOv.IL-3 (Fig. 6.3). SMCP-positive cells were detected immunohistochemically (Fig. 6.4(a)). They were categorised as putative mast cells represented by both small and large mononuclear cells (Fig. 6.4(b)), which were distinct from a small number (~ 1% to 2% of the total cell population) of basophil-like SMCP-positive

polymorphonuclear cells (Fig. 6.4(c)). The percentage of SMCP-positive mast cells generated on days 12 and 17 of culture was also a function of the concentration of rOv.IL-3 (Fig. 6.5). The numbers of SMCP-positive mast cells were increased ($P<0.05$-0.01; Fig. 6.5) when grown in the presence of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} rOv.IL-3 on day 12 , and in 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} rOv.IL-3 on day 17. When both the total viable cell count and the percentage of SMCP-positive mast cells was considered (Figs. 6.1 and 6.5), the greatest numbers of mast cells were generated in the presence of the higher doses of rOv.IL-3 (particularly 10^{-1} rOv.IL-3 on days 12 or 17).

The concentration of SMCP per well was increased over control values in the presence of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} rOv.IL-3 on both days 12 and 17 ($P<0.05$-0.01; Fig. 6.6(a)). This effect was dose-dependent and probably reflected the higher percentage of SMCP-positive mast cells and the increased viable cell count that resulted when cells were grown in higher concentrations of rOv.IL-3.

Arylsulfatase and β-hexosaminidase activity in rOv.IL-3 supplemented wells increased in an apparently dose-responsive fashion only on day 17, but owing to a lack of control sample measurements at this time point (insufficient sample following repetition of original SMCP ELISA) this data could not be evaluated statistically (Fig. 6.6 (b) and (c)).

The results of this experiment demonstrated that supplementation of IMDM/10% FCS with rOv.IL-3 increased the total viable cell count of the culture, and favoured the development of a population of putative BMMC. Arylsulfatase and β-hexosaminidase activities were also demonstrated in the cultured cell population, although the source of this activity was not identified. The peak viable cell count on day 17 was obtained with 10^{-1} rOv.IL-3 (9.9 ± 1.2 $x10^5$ cells/ml; Mean ± SEM, n=3; Fig. 6.1), this cell population consisting of 35.8 ± 6.7 %

(Mean ± SEM, n=3) mast cells (Fig. 6.5). Therefore, of the concentrations tested, 10^{-1} rOv.IL-3 was considered to generate the greatest numbers of BMMC *in vitro*.

The dose response experiment was repeated with concentrations of 10% (equivalent to 10^{-1}), 20% and 30% rOv.IL-3, harvesting occurring at days 8, 14 and 17 (Appendix B, Fig. B.1 and Fig. B.2). This confirmed that a concentration of 10^{-1} rOv.IL-3 generated the greatest numbers of mast cells in culture. However, it was evident that variability existed between the two cultures in terms of both the useful life-span of the cultures and the maximum percentage of mast cells obtained.

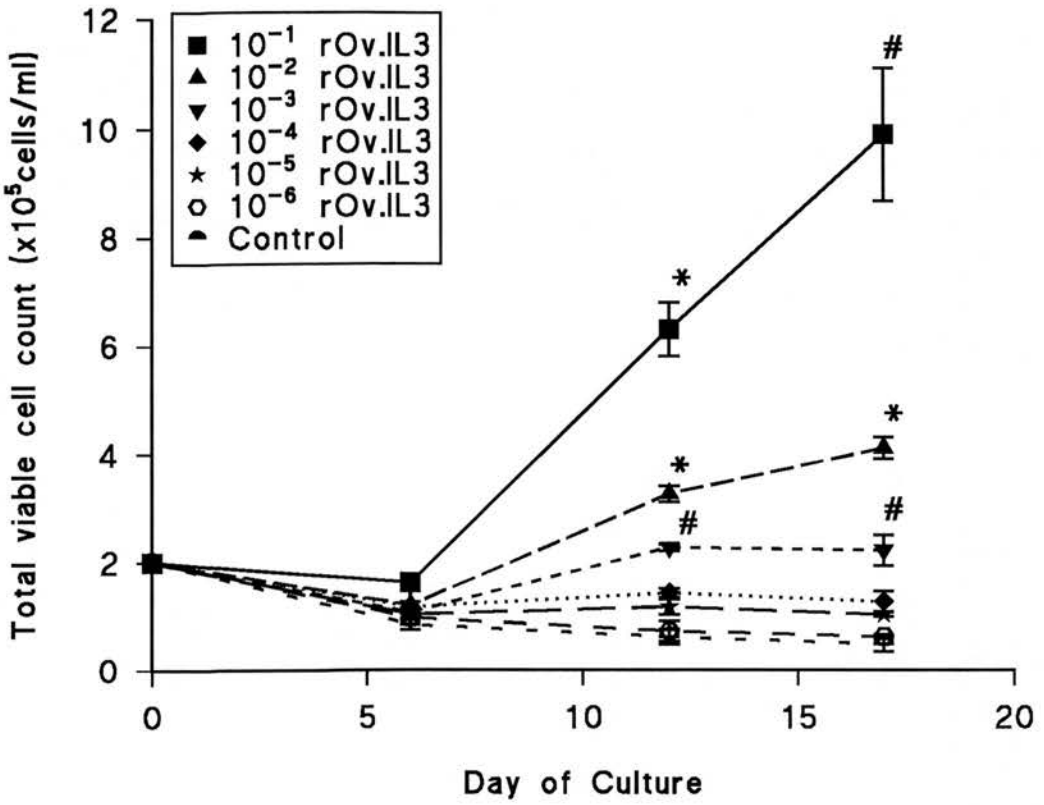


Figure 6.1. Growth of bone marrow cells shown as total viable cell count (x10^5 cells/ml) plotted against time (days) for different dilutions of rOv.IL-3 (Mean ± SEM; n=3). # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$.

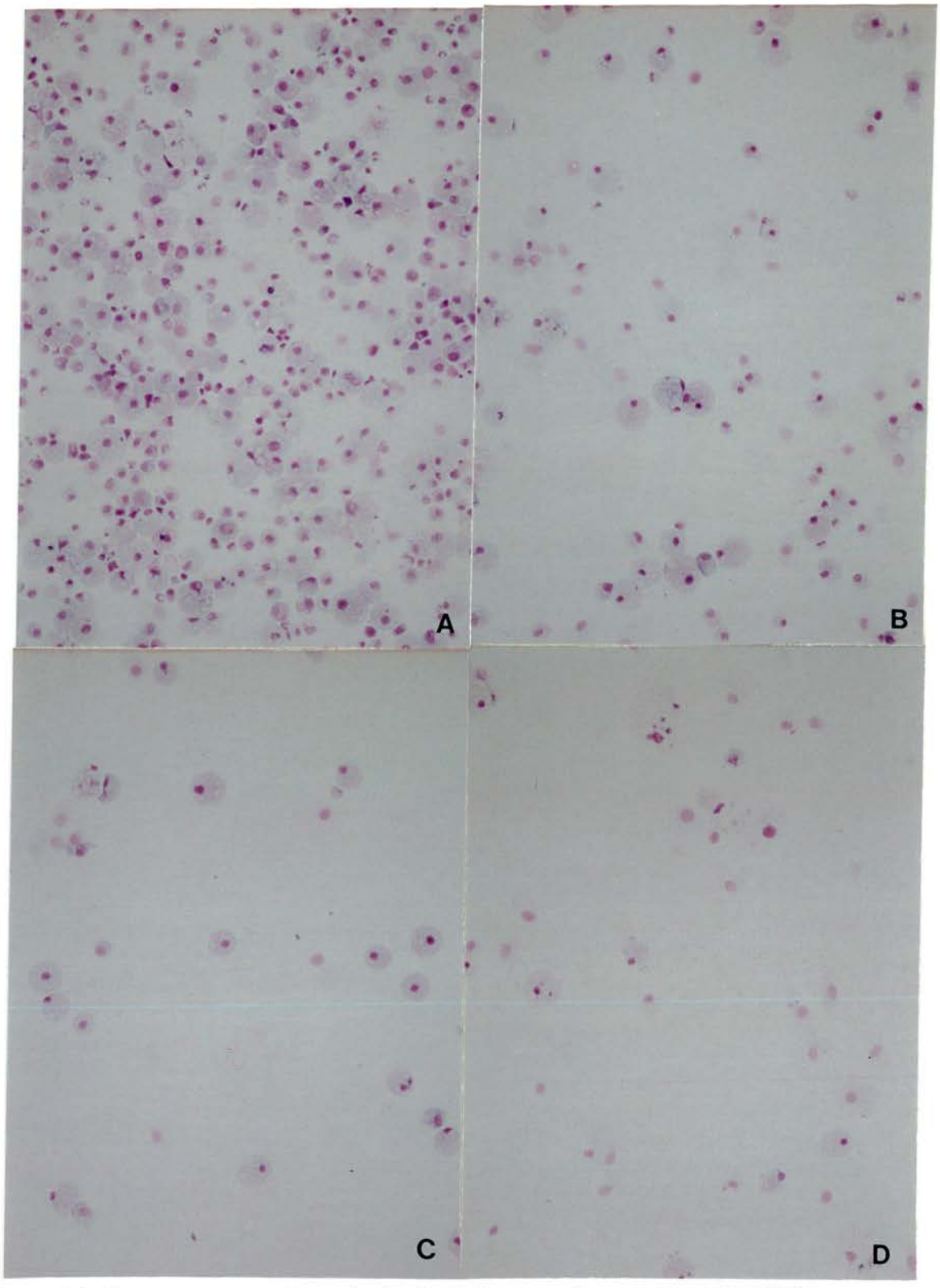


Figure 6.2. Cytosmear preparations of ovine bone marrow cell suspension grown in the presence of 10^{-1} **(a)**, 10^{-3} **(b)**, 10^{-6} **(c)** rOv.IL-3 and in the presence of IMDM/10% FCS alone **(d)** at day 17 of culture (Leishman's stain, x312.5). Each cytosmear was prepared with 125µl of cell suspension.

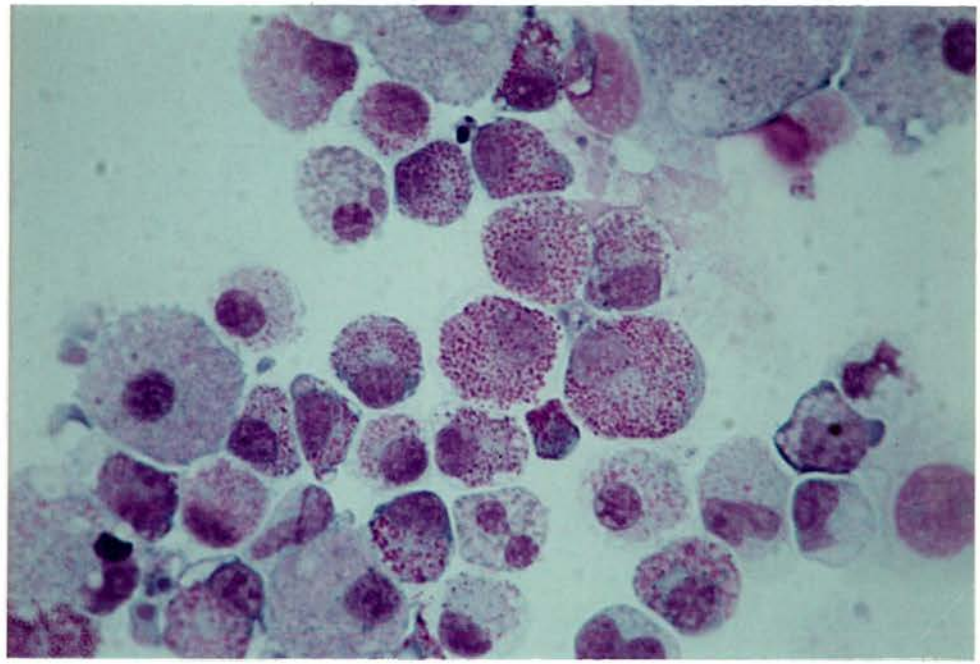
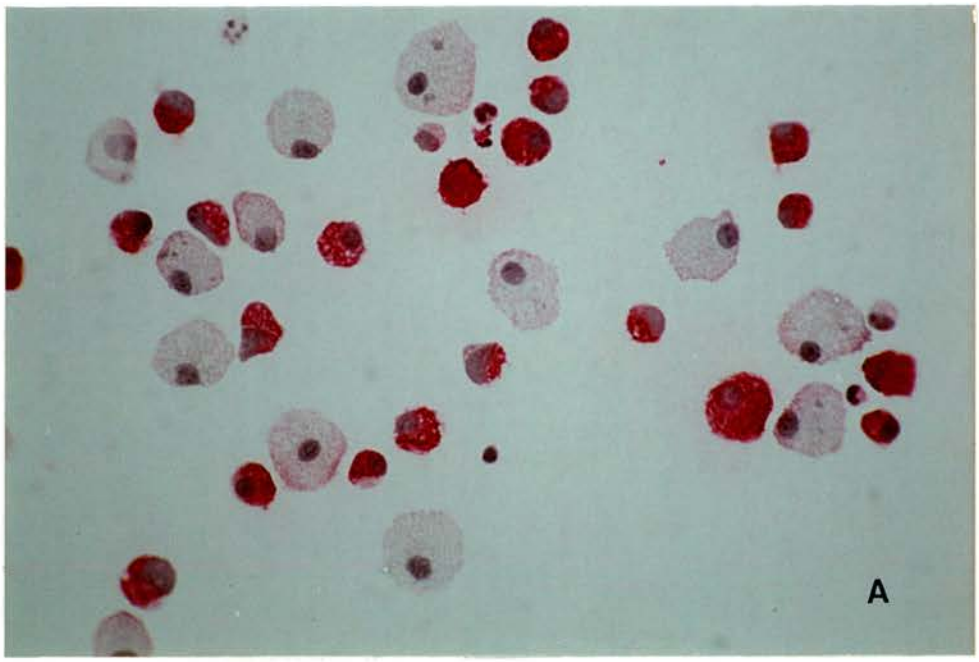


Figure 6.3. Leishman's-stained cytosmear preparation of ovine bone marrow cells grown in the presence of 10^{-1} rOv.IL-3 at day 12 of culture. Note large numbers of mononuclear, granulated mast cells (x625).



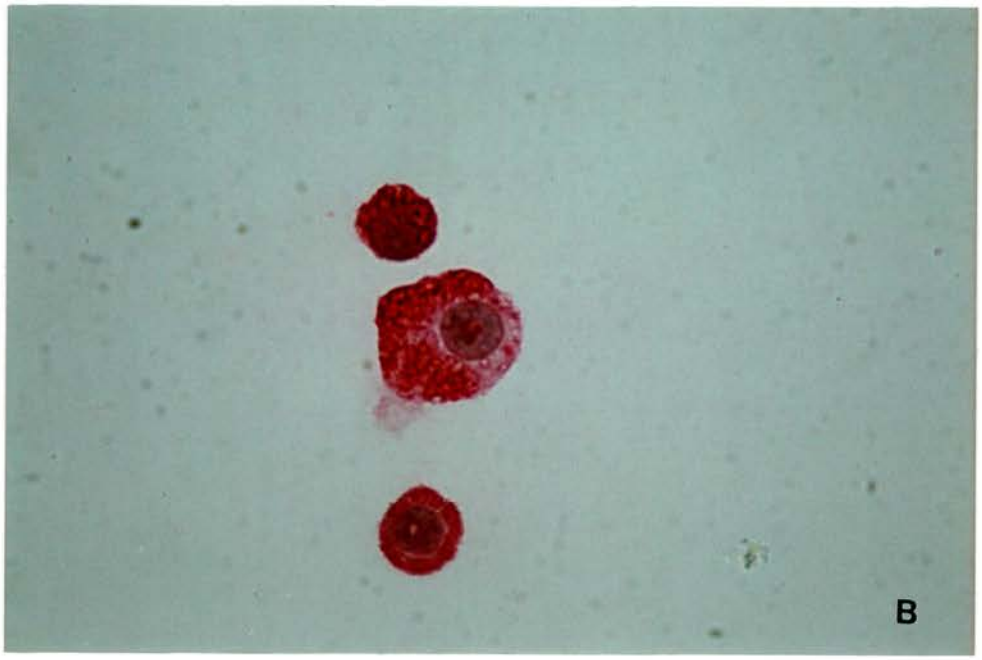


Figure 6.4(a) and (b). Cytosmear preparations of ovine bone marrow cells grown in the presence of 10^{-1} rOv.IL-3 on day 12 of culture stained immunohistochemically with 2.5µg/ml PBS of affinity purified polyclonal rabbit anti-SMCP (Batch 148/93) as the primary antibody (2.3.6, 2.3.8.2). **(a)** : Note the intense red staining indicating the presence of SMCP and the absence of staining of fibroblast-like cells (x312.5). **(b)** : Small and large putative mast cells (mononuclear), staining intensely for SMCP (x625). All control cytosmears were negative (not shown).

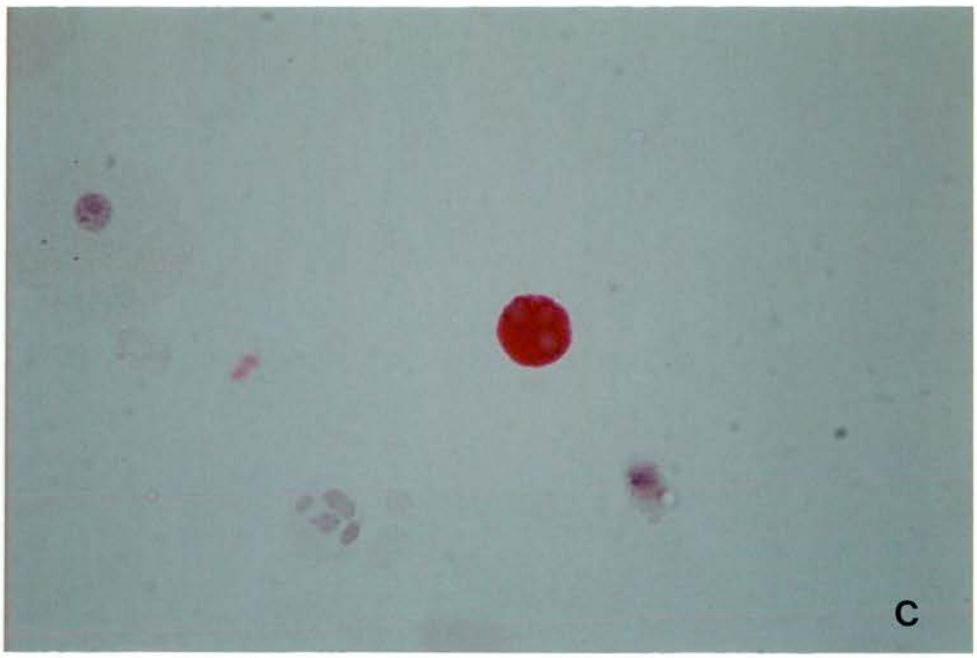


Figure 6.4(c). Cytosmear preparations of ovine bone marrow cells grown in the presence of 10^{-1} rOv.IL-3 on day 12 of culture stained immunohistochemically with 2.5µg/ml PBS of affinity purified polyclonal rabbit anti-SMCP (Batch 148/93) as the primary antibody (2.3.6, 2.3.8.2). **(c)** : Basophil-like cell staining intensely for SMCP (x625). All control cytosmears were negative (not shown).

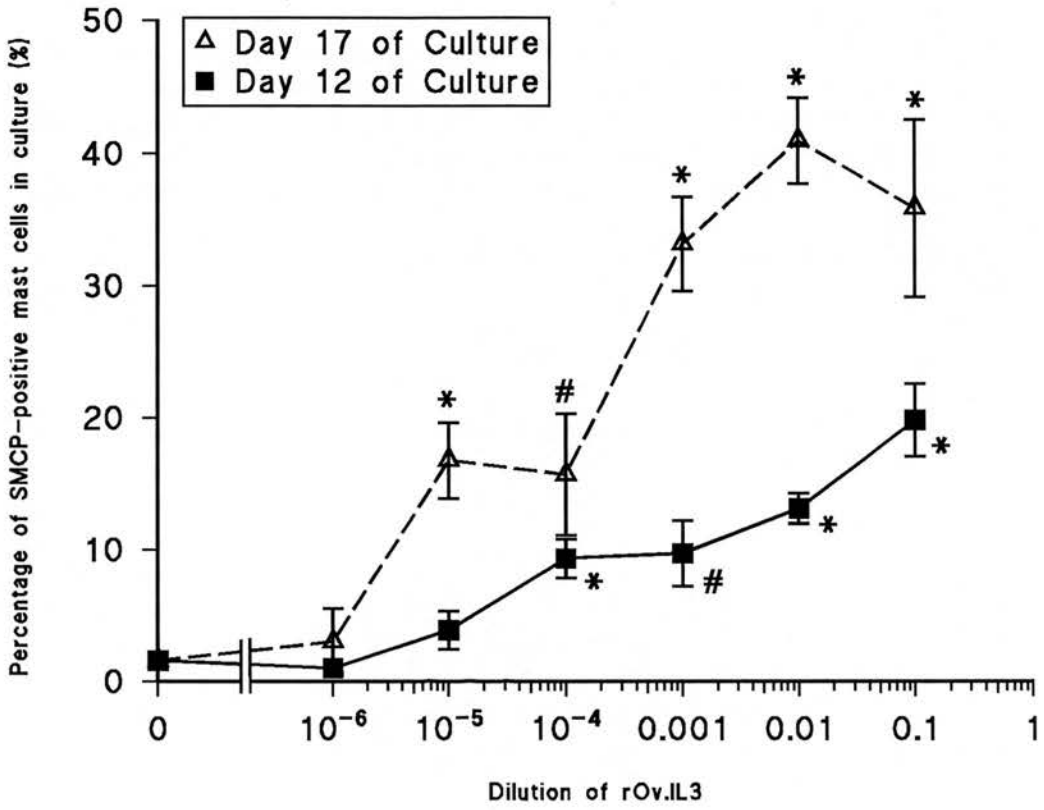


Figure 6.5. The effect of rOv.IL-3 on the growth of BMMC. The percentage of SMCP-positive mast cells is plotted against the dilution of rOv.IL-3 at days 12 and 17 of culture (Mean ± SEM; n=3). # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$.

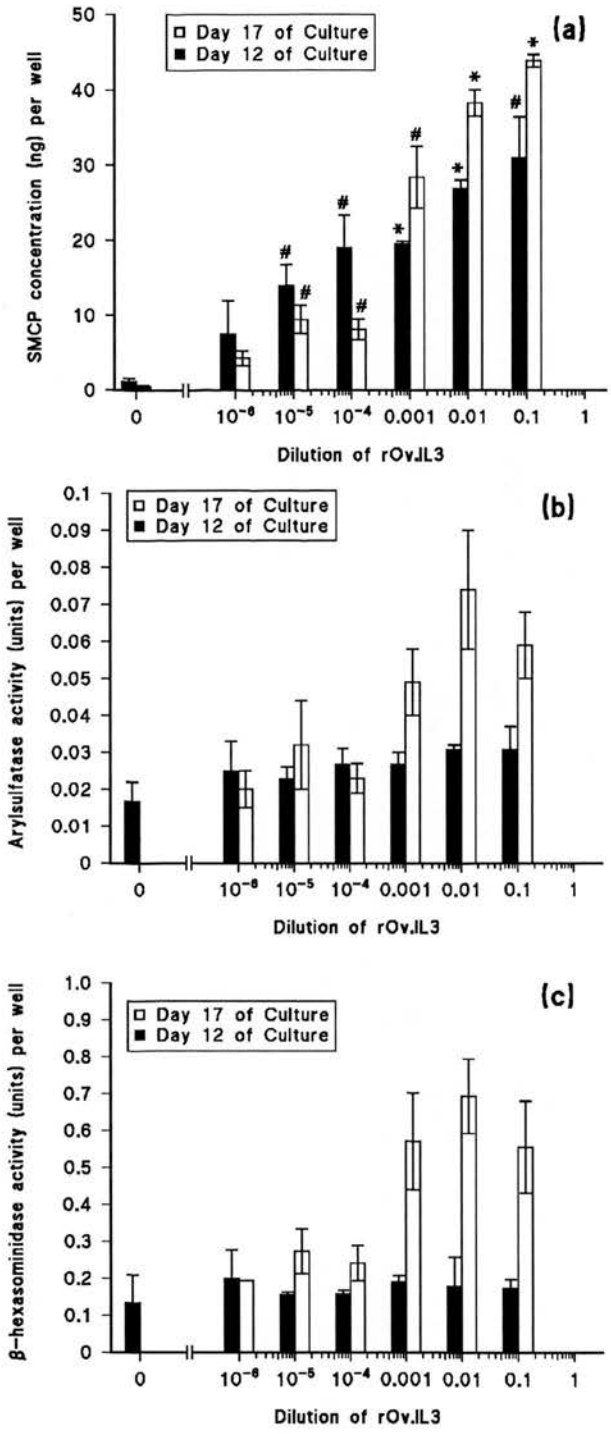


Figure 6.6. The concentration of SMCP (ng/well) (**a**), and activities (units/well) of arylsulfatase (**b**) and β-hexosaminidase (**c**) on days 12 and 17 of culture (Mean ± SEM; n=3). As described in the text, no control samples were available for arylsulfatase and β-hexosaminidase assay at day 17. # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$.

6.2.2. Time Course Study

Using the optimal concentration of 10^{-1} rOv.IL-3, the kinetics of growth and development of ovine BMMC were investigated. Test and control cultures were plated out in triplicate in 24 well plates, cells being harvested at days 2, 5, 7, 9, 12, 14, 16 and 19.

6.2.2.1. Results

An increase in the total viable cell count in the presence of 10^{-1} rOv.IL-3 was seen from day 5 of culture, and reached a peak at day 16 ($P<0.05$-0.01; Fig. 6.7). The percentage of SMCP-positive mast cells increased in both test and control groups on day 2 of culture, before falling again in both groups by day 5 (Fig. 6.8). A further rise only in rOv.IL-3 supplemented wells plateaued on days 14-16 ($P<0.01$ on days 9, 12, 14, 16 and 19; Fig. 6.8), the subsequent decline in SMCP-positive cells (Fig. 6.8) being coincident with the decline in viable cell count between days 16 and 19 (Fig. 6.7).

Concentrations of SMCP in both test and control wells followed similar kinetics to that described for SMCP-containing cells with an initial peak on day 2, followed by a decline and subsequent increase, with maximum concentrations occurring on days 12 to 16 ($P<0.05$-0.01; Fig. 6.9(a)).

In contrast, the activity of both arylsulfatase and β-hexosaminidase increased gradually from day 0 of culture ($P<0.05$-0.01 on days 9, 12, 14 and 16 for arylsulfatse [Fig. 6.9(b)] and $P<0.05$-0.01 on days 7, 9, 12 and 14 for β-hexosaminidase [Fig. 6.9(c)]). Increased activity in control wells (Fig. 6.9(b) and Fig. 6.9(c)) with low viable cell counts and low percentages of SMCP-positive mast cells would suggest that these mediators were also present in cells other than mast cells. Using linear regression analysis, the concentration or activity of each mediator in each individual well was correlated to either the cell total or to the number of SMCP-positive mast cells present in each well (Table 6.1). Highly significant correlations

($P<0.0001$) were demonstrated for each mediator with *both* cell populations. Thus, the cellular source of mediator could not be identified from these results. However, it is of interest that the r value for SMCP is apparently greater for the SMCP-positive mast cell population (0.76) than for the general cell population (0.60). Conversely, the r values for both arylsulfatase and β-hexosaminidase are apparently greater when correlated with the general cell population than with the SMCP-positive mast cell population.

In an effort to further characterize the mast cells generated in the presence of 10^{-1} rOv.IL-3, electron microscopy was undertaken on cells harvested at day 13 from a separate culture (2.3.16). Eight separate grids were examined, with approximately 40 mast cells being present per grid. A representative mast cell is shown in Fig. 6.10. The cells were round or oval, mononuclear, with a sparse complement of cytoplasmic granules. The granules present contained little electron-dense material. The endoplasmic reticulum and Golgi apparatus were not well developed in any of the cells examined. Pseudopodia were a prominent and consistent feature in all cells.

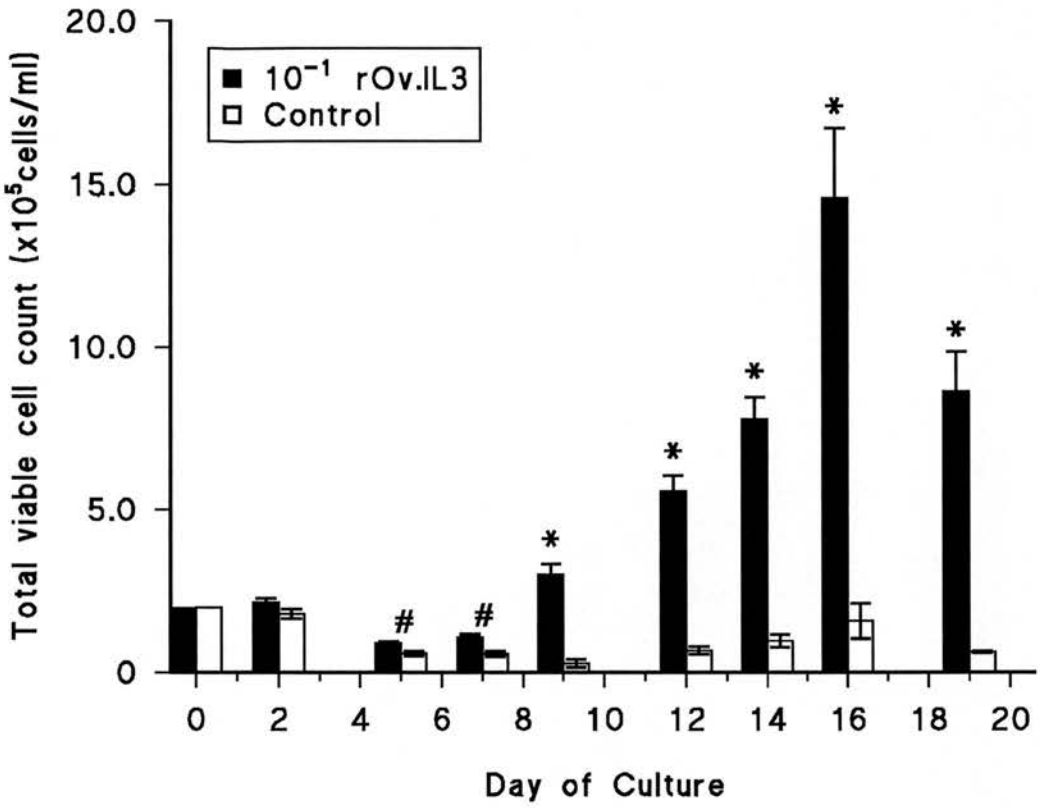


Figure 6.7. Growth of ovine bone marrow cells shown as the total viable cell count (x10^5 cells/ml) plotted against time (days) (Mean ± SEM; n=3). # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$.

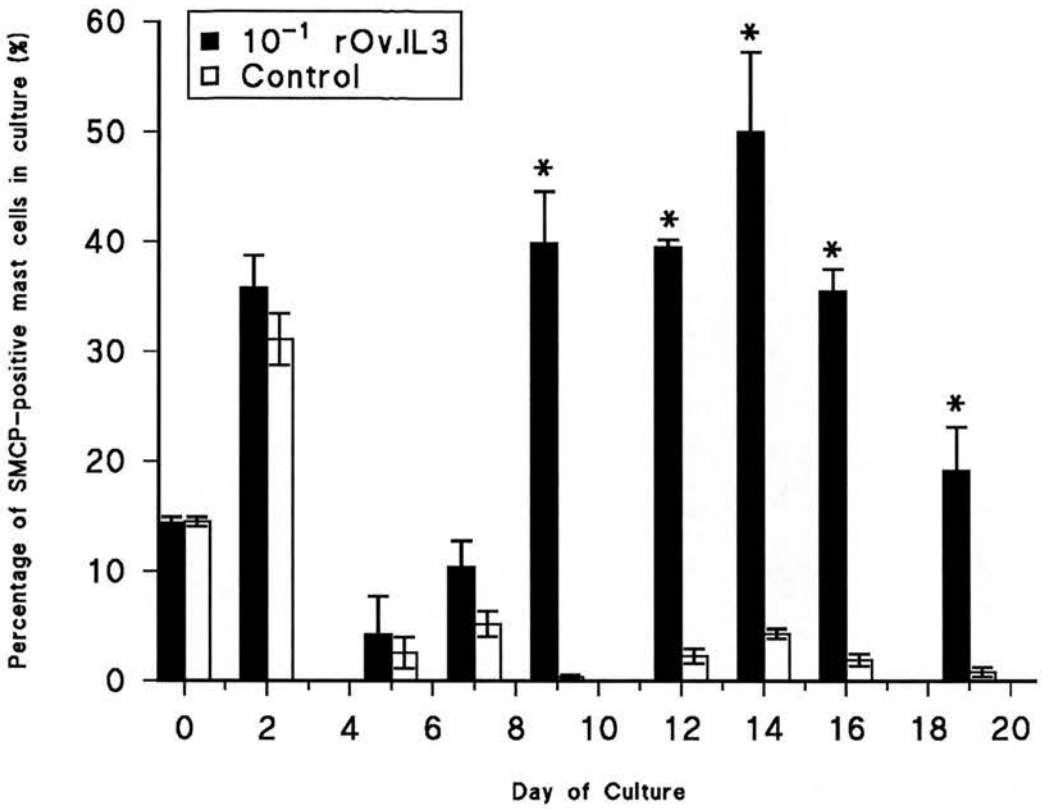


Figure 6.8. The effect of time on the growth and development of BMMC. The percentage of SMCP-positive mast cells is plotted against time (days) (Mean ± SEM; n=3). * $P<0.01$ compared with IMDM/10% FCS control.

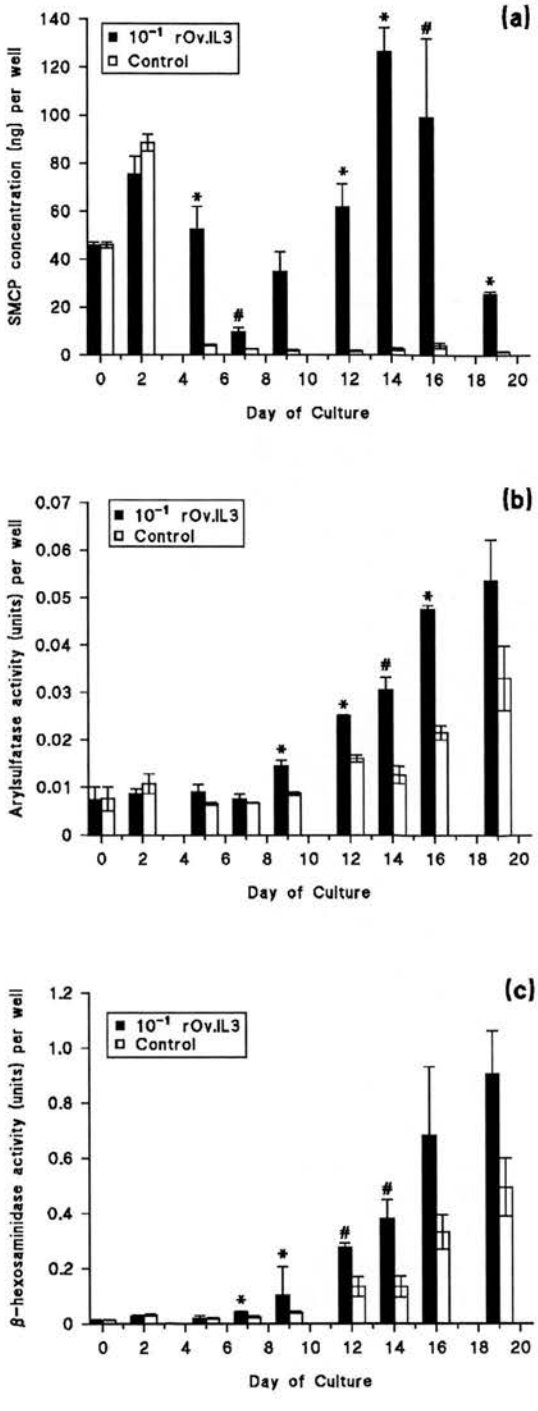


Figure 6.9. The concentration of SMCP(ng/well) **(a)**, and activities (units/well) of arylsulfatase **(b)** and β-hexosaminidase **(c)** plotted against time (days) (Mean ± SEM; n=3). # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$.

Mediator	Correlation with the absolute number of cells in the general cell population of the well (r =).	Correlation with the absolute number of SMCP-positive mast cells present in the well (r =).
SMCP	0.60 (P<0.0001)	0.76 (P<0.0001)
Arylsulfatase	0.78 (P<0.0001)	0.68 (P<0.0001)
β-hexosaminidase	0.70 (P<0.0001)	0.58 (P<0.0001)

Table 6.1. Mediator content or activity of the cell population of each well correlated to the total cells per well or to the number of SMCP-positive mast cells per well.

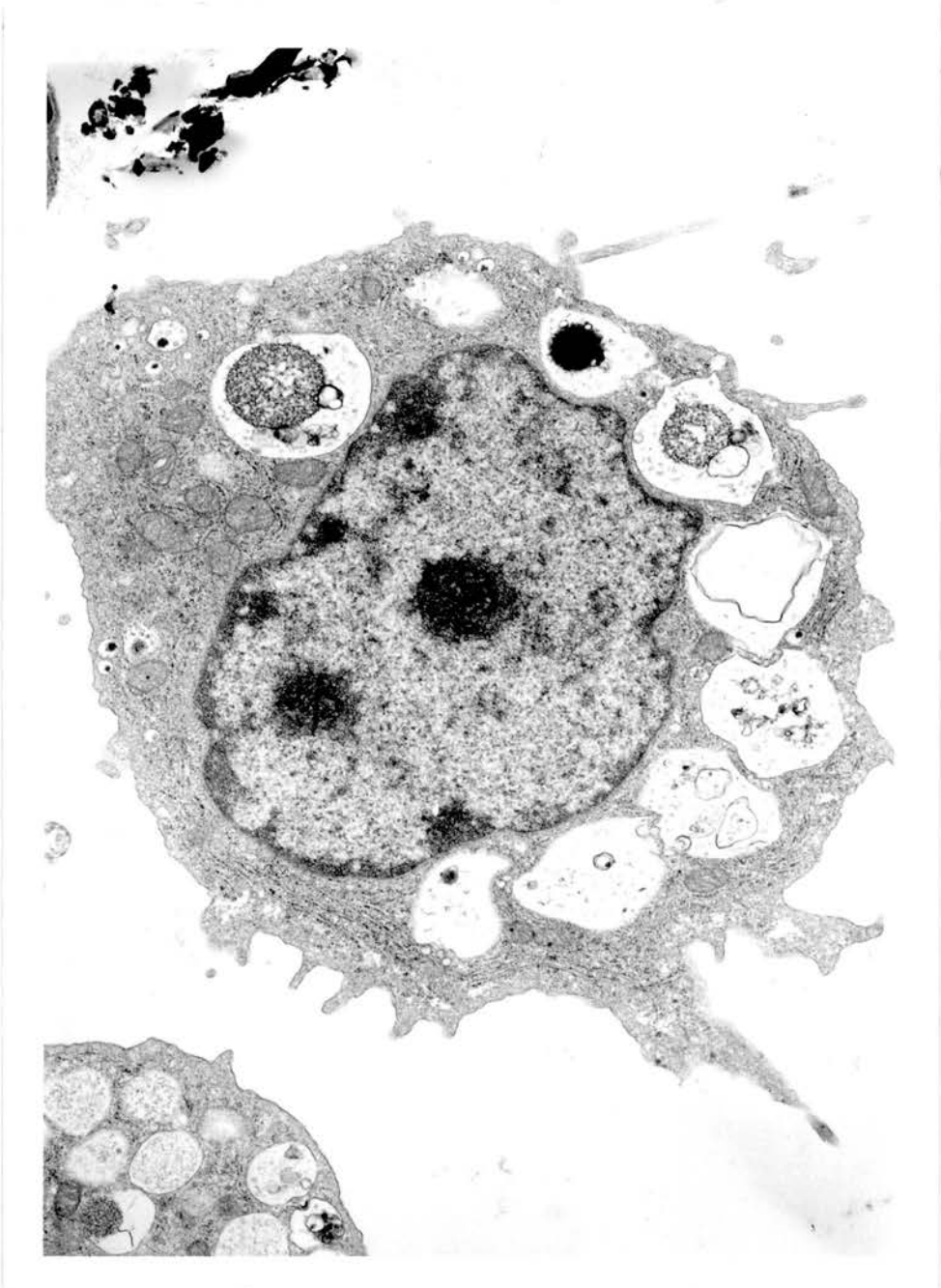


Figure 6.10. Electron photomicrograph of an ovine rOv.IL-3-dependent BMMC at day 13 of culture. Note mononuclear appearance and relative paucity of granules. Prominent pseudopodia are present. (x 12000).

6.2.3. Removal Of Adherent Cells

In an attempt to increase the maximal percentage of SMCP-positive mast cells obtained in culture, the effect of transferring non-adherent cells at feeding was investigated. The hypothesis was that such transfer would result in depletion of the adherent fibroblast-like cells. Using 10^{-1} rOv.IL-3 bone marrow cells were plated out into two 24-well plates. In plate I, the cell population was fed at days 6 and 14, but retained in the original plate. In plate II, non-adherent cells were transferred to wells in a fresh plate at feeding. Cell harvests from both plates were undertaken at days 14 and 20.

6.2.3.1. Results

An increase in the percentage of SMCP-positive mast cells in rOv.IL-3 supplemented wells was observed in plate II in comparison to plate I on both days 14 ($P=0.05$) and 20 ($P=0.04$) (Fig. 6.11). Given that there was no significant difference in the viable cell count for rOv.IL-3 supplemented wells at day 14 between these plates ($P=0.75$; Fig. 6.12), the greatest total number of BMMC would be obtained by harvesting plate II. However, by day 20, there was a statistically significant reduction in viable cell count for rOv.IL-3 supplemented wells in plate II in comparison to plate I. Thus, the optimal time for cell harvesting had passed by day 20. (There were no significant differences in viable cell count for control wells between plates I and II at all time points studied, nor when comparing rOv.IL-3 supplemented wells in plates I and II at day 6).

This result demonstrates that the maximal percentage of SMCP-positive mast cells generated *in vitro* could be increased by transferring the non-adherent cell population to fresh wells at feeding (Fig. 6.11). Additionally, if the viable cell count data is also considered (Fig. 6.12), a greater *absolute* number of mast cells is obtained if previously transferred cells are harvested at day 14 of culture.

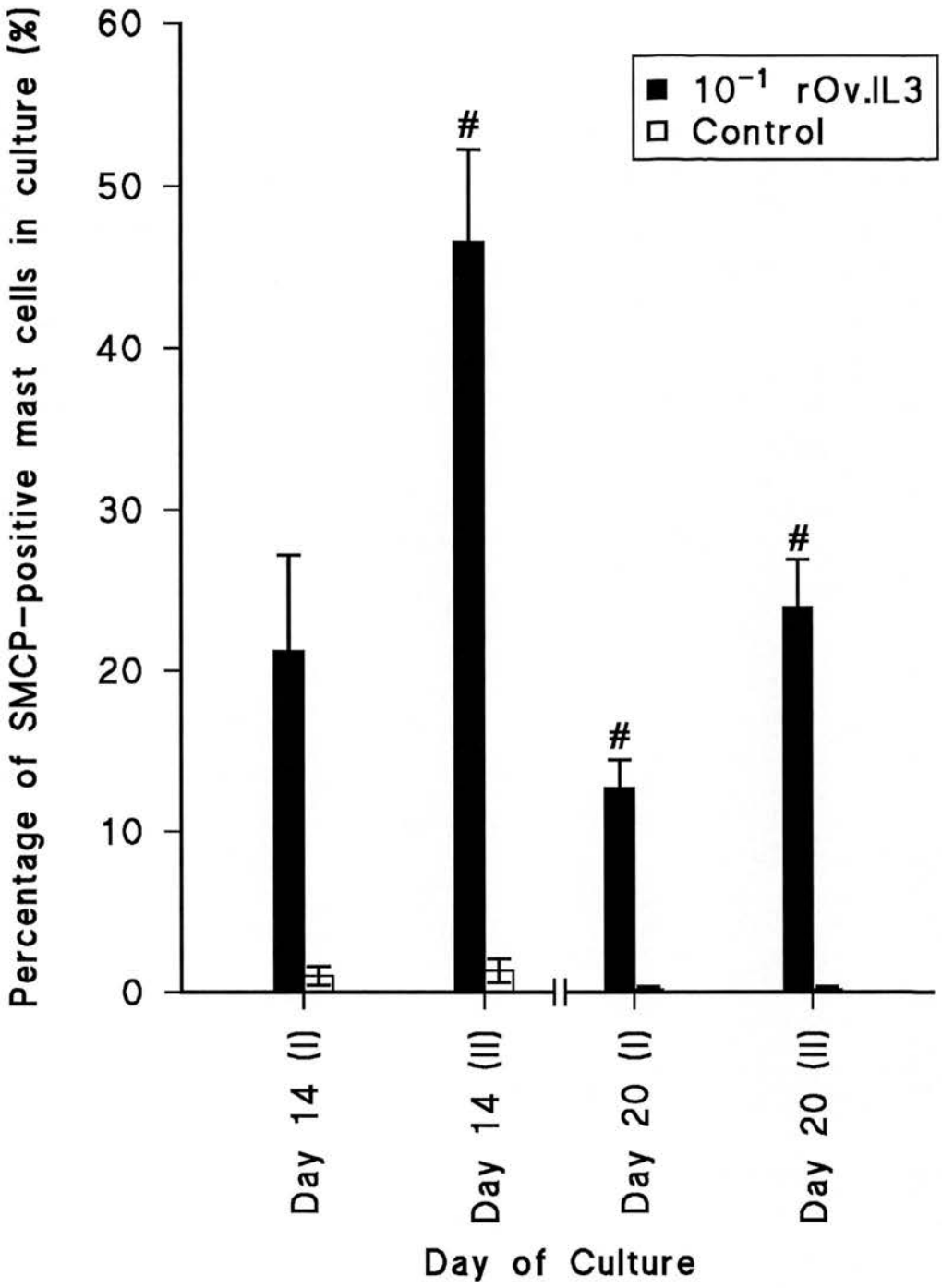


Figure 6.11. The effect of transferring non-adherent bone marrow cells on the growth of BMMC. The percentage of SMCP-positive mast cells for plates I (non-transferred) and II (transferred) is plotted against time (days) (Mean ± SEM; n=3). Plates were fed (and non-adherent cells in plate II transferred) at days 6 and 14 of culture. # $P<0.05$ compared with IMDM/10% FCS control.

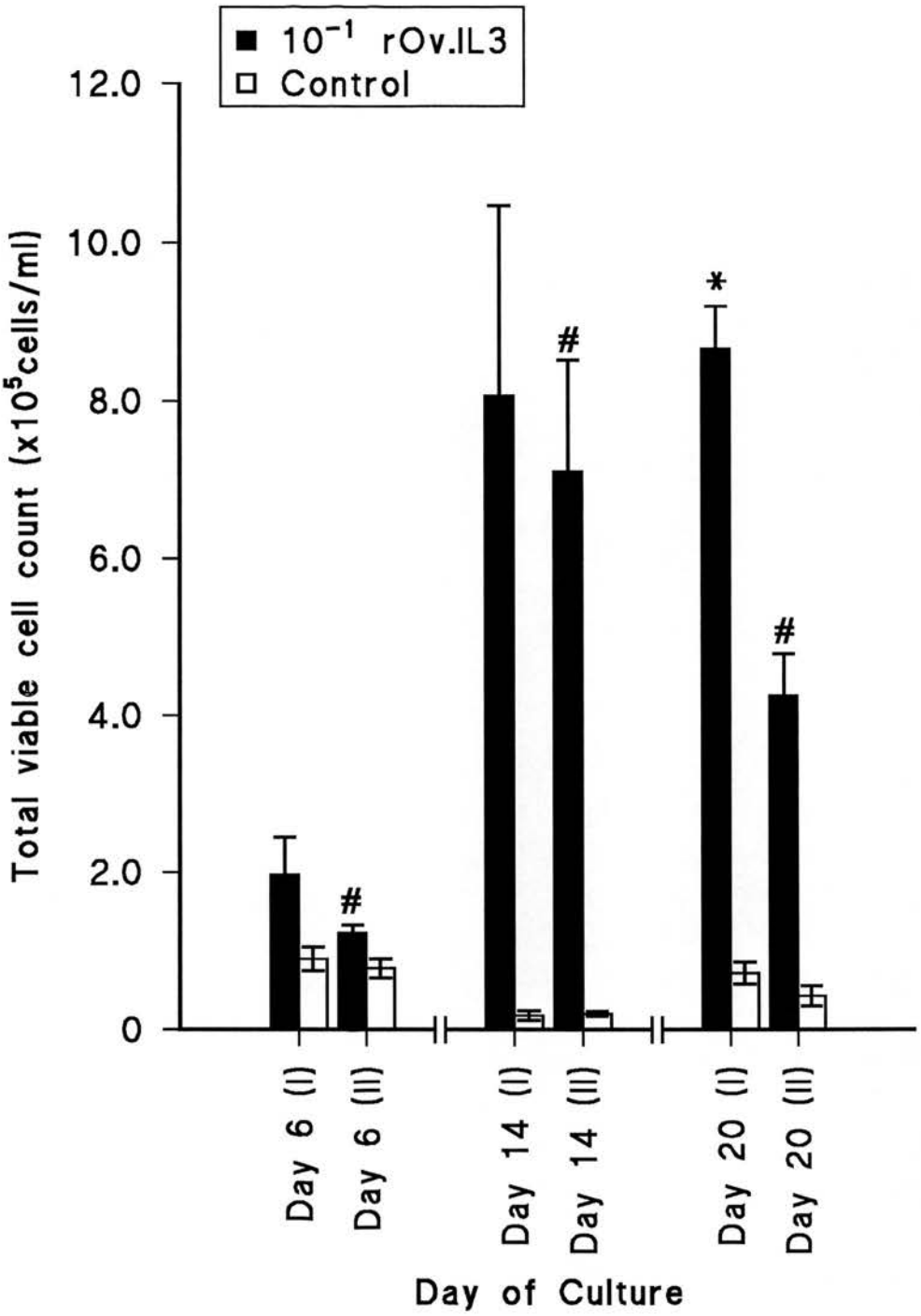


Figure 6.12. The effect of transferring non-adherent bone marrow cells on cell growth. The total viable cell count (x10^5 cells/ml) for plates I (non-transferred) and II (transferred) is plotted against time (days) (Mean ± SEM; n=3). Plates were fed (and non-adherent cells in plate II transferred) at days 6 and 14 of culture. # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$.

6.3. Discussion

Marked differences were observed in the final quality of the rOv.IL-3 BMMC culture obtained in comparison to IL-3-dependent mast cell populations derived from the mouse (Ghiara et al 1985, Rennick et al 1985, Chiu and Burrall 1990) and rat (Haig et al 1988a). Firstly, rOv.IL-3 BMMC cultures were relatively short lived, with cell viability declining after approximately two weeks in culture. This contrasts with the rat (Haig et al 1988a) and mouse (Chiu and Burrall 1990), where IL-3-dependent BMMC cultures were stable for up to 6 weeks and 5 months respectively. Secondly, the maximum percentage of rOv.IL-3 BMMC obtained in any of the experiments was 72.5 ± 4.0% (Appendix B, Fig. B.2.; 20% rOv.IL-3 harvested at day 8 of culture). However, mast cell populations of >90% purity are achieved within 14 days in both mouse and rat (Chiu and Burrall 1990, Haig et al 1988a). Additionally, when ovine bone marrow cells are grown in the presence of lymphocyte-derived conditioned medium (CM), cultures consisting of 90% BMMC are attained by day 28, and survive for up to 3 months (Haig et al 1988b).

Electron microscopy of these CM-derived BMMC (CM-BMMC) reveals more complete granule formation (Huntley et al 1992) than in comparable rOv.IL-3 BMMC (Fig. 6.10), even though these CM-BMMC are themselves more poorly granulated than mucosal mast cells (MMC) from parasitized abomasum (Huntley et al 1992). As rOv.IL-3 can also act synergistically with CM to produce increased numbers of mast cell colonies in soft agar bone marrow cell clonogenic assays (Haig 1993), it is likely that other cytokines or factors are necessary to support the proliferation, differentiation and ultimate maturation of ovine rOv.IL-3-dependent BMMC *in vitro*. It would therefore appear that IL-3 is not the sole factor supporting mast cell-growth in ovine conditioned medium.

The rOv.IL-3 BMMC were SMCP-positive, which is consistent with results for CM-BMMC (Haig et al 1988b, Huntley et al 1992). The rOv.IL-3 BMMC were not examined for the presence of an additional serine proteinase present in CM-BMMC but not in abomasal MMC (Huntley et al 1992). The hypothesis that CM-BMMC may contain a mixture of MMC and CTMC phenotypes (Huntley et al 1992) could not, therefore, be extended to rOv.IL-3 BMMC. However, given the results from Chapter 3 that the MMC of the ovine gastrointestinal tract are overwhelmingly SMCP-positive (putative ovine MMC phenotype) and that the majority of mast cells in the skin are SMCP-negative (putative ovine CTMC phenotype), then this would suggest that rOv.IL-3 BMMC resemble the putative ovine MMC phenotype. In the rat, IL-3 stimulates the growth of a population of BMMC almost identical to MMC (Haig et al 1988a) that are homogeneous in proteinase phenotype (Haig et al 1988a). In contrast, IL-3-dependent murine BMMC can express a non-MMC phenotype, expressing both MMCP-5 (M^{c}Neil et al 1991) and MMCP-6 (Reynolds et al 1991). Indeed, murine BMMC are considered a heterogeneous cell population with regard to their proteinase content (Newlands et al 1991), with some cells expressing a serosal mast cell phenotype. Mast cell proteinase expression can be altered by a range of cytokines (reviewed in Chapter 1). For example, IL-9 can enhance the proliferation of murine mast cell lines induced by IL-3 or IL-4 (Hültner et al 1989, Moeller et al 1989, Hültner et al 1990), the latter cytokines also inhibiting the IL-9-induced expression of MMCP-1, MMCP-2 and MMCP-4 (Eklund et al 1993). Importantly, stem cell factor (SCF) was shown to act synergistically with IL-3 to promote the growth of both rat (Haig et al 1994) and mouse (Tsai et al 1991a) IL-3-derived BMMC. *In vivo*, SCF was shown to induce the development of both CTMC and MMC at the appropriate anatomical sites in the rat (Tsai et al 1991b). Therefore, it may be that combinations of cytokines (particularly SCF in addition to IL-3) are required to generate ovine BMMC that would be phenotypically analogous to the predominantly SMCP-negative dermal mast cell

population. However, during the course of these studies, recombinant ovine SCF was not available.

The biphasic peak of both SMCP-positive mast cells and SMCP concentration demonstrated in the time course study warrants further investigation. SMCP-positive cells are present in day 0 bone marrow, and it may be that these cells comprise two subpopulations. Firstly, a subpopulation of committed progenitor cells initially stimulated by the bone marrow microenvironment may rapidly mature in the presence of exogenous IL-3, resulting in the initial section of the biphasic peak, these cells then rapidly dying. Secondly, a population of stem cells may differentiate, proliferate and mature gradually from day 0 in the presence of exogenous IL-3 to give the second phase of the biphasic peak at approximately day 14. This hypothesis is complicated by the presence of apparently mature, SMCP-containing basophil-like cells. No basophil-like cells were reported in ovine CM-BMMC cultures (Haig et al 1988b, Huntley et al 1992). In both mouse (Rottem et al 1993) and man (Kirshenbaum et al 1989, Valent et al 1989) basophils can be generated from bone marrow cells grown in the presence of IL-3, which would favour the rOv.IL-3 BMMC population being more like mouse BMMC than rat BMMC (no mature basophils being generated in this system; Haig et al 1988a). It is acknowledged that immature basophils can be difficult to differentiate morphologically from mast cells (Dvorak 1986), and that these cells may have been classified in the early stages of culture as mast cells, prior to developing a fully polymorphonuclear appearance. Thus, these immature cells could have been erroneously included in cell counts contributing to the magnitude of the initial phase of the biphasic peak.

Unlike SMCP (where immunohistochemistry could be readily performed on BMMC), it could not be stated with certainty that ovine rOv.IL-3 BMMC contained arylsulfatase and β-hexosaminidase, owing to their possible production by other cell types in the cultures.

However, with this in mind, when time course study results for rOv.IL-3 BMMC at day 19 (the latest time point available for comparison) are expressed in terms of the quantity of SMCP or activities of arylsulfatase or β-hexosaminidase per 10^6 mast cells, the results are similar to those reported for CM-BMMC harvested between days 21 and 35 of culture (Huntley et al 1992; Table 6.2). The figures for CM-BMMC were derived from cultures that included between 10% and 30% of contaminating cell types, so the query regarding the cellular source of arylsulfatase and β-hexosaminidase is still pertinent. Had time allowed, an attempt could have been made to identify the cellular source of arylsulfatase and β-hexosaminidase in mast cell cultures using dual immunohistochemistry on adjacent thin sections (the arylsulfatase subtype present in the rOvIL-3 BMMC population was not defined in these studies). The concentration of SMCP in rOv.IL-3 BMMC is approximately half of that in CM-BMMC, which may reflect the granule immaturity of rOv.IL-3 BMMC. Mature MMC isolated from the abomasum (Huntley et al 1992) contain considerably more SMCP than either rOv.IL-3 BMMC or CM-BMMC (Table 6.2). Therefore, in terms of their content of granule proteinase, rOv.IL-3 BMMC can be considered again similar to murine BMMC (Newlands et al 1991) and unlike rat IL-3 BMMC (Haig et al 1988a), in that they contain very low levels of granule proteinase.

Arylsulfatase activity appears similar between the two ovine BMMC populations, and approximates that of abomasal MMC. Interestingly, both rOv.IL-3 BMMC and CM-BMMC appear to contain more β-hexosaminidase activity than do abomasal MMC, with rOv.IL-3 BMMC containing approximately $2^1/_2$ times the activity of CM-BMMC. However, until the contribution of the contaminating cell population is known, or until a pure ovine rOv.IL-3 BMMC population can be generated, further interpretation of these results is speculative.

Mast cell activation during mediator release studies can be assessed by the release of arylsulfatase and β-hexosaminidase (Stevens et al 1986a, Broide, Metcalfe and Wasserman 1988, MacDonald et al 1989, Huntley et al 1992), owing to their localisation to the mast cell secretory granule (Schwartz et al 1981b). However, in the rOv.IL-3 BMMC culture where contaminating cells are present, the possible activation of these cells by non-mast cell-specific secretagogues (e.g. calcium ionophore A23187) must be considered. Additionally, the presence of a small population of basophil-like cells (Fig. 6.4(c)) also complicates release studies, as it is not known if these would be capable of activation by chemical secretagogues (Foreman 1993). Interpretation of release studies (Chapter 7) must therefore be undertaken with caution, although it can be inferred that for rOv.IL-3 BMMC the release of SMCP rather than arylsulfatase or β-hexosaminidase may provide a more valid and reliable indicator of specific mast cell activation.

Given that culture conditions were constant the variability in cell viability and maximum percentage of mast cells probably reflects between sheep variability. Presumably, such variations may result from changes in the initial combination of microenvironmental factors (e.g. cytokines) present in bone marrow that can directly regulate the number and type of stem or progenitor cells, thereby affecting the number of cells subsequently committed to proliferation and differentiation into mast cells (Metcalf 1993).

The series of experiments described in this chapter successfully defined the optimal conditions for generation of rOv.IL-3-dependent BMMC in a liquid culture system. An IMDM/10% FCS medium (Appendix A) containing a final dilution of 10^{-1} rOv.IL-3 was found to be optimal. The non-adherent cell population should be transferred to fresh plates at feeding, with cell harvesting occurring between days 12 and 16 of culture. This protocol was therefore

employed for large scale generation of rOv.IL-3 BMMC in flasks for use in mediator release studies (Chapter 7).

The initial characterisation of these rOv.IL-3 BMMC could therefore be further investigated. This prompted the mediator release studies described in Chapter 7, where the functional activity of these putatively MMC-like cells was elucidated using substance P, compound 48/80, calcium ionophore A23187 and SMCP as putative secretagogues. These results could be compared with the effects of the same agents in ovine skin *in vivo* (Chapters 4 and 5), where the majority of mast cells are SMCP-negative (the putative CTMC phenotype; Chapter 3). Additionally, given that there are differences in the nature and development of the cell populations generated by rOv.IL-3 and CM, the functional activity of rOv.IL-3 BMMC in terms of mediator release could be compared with that reported for CM-BMMC. These investigations would further define ovine mast cell heterogeneity.

	Concentration per 10^6 mast cells		
Mediator	**IL-3 BMMC**	**CM-BMMC***	**Abomasal MMC***
SMCP (ng)	**212.6 ± 36.4**	**485 ± 154**	**3875 ± 563**
Arylsulfatase (units of activity)	**0.42 ± 0.02**	**0.43 ± 0.04**	**0.88 ± 0.47**
β-hexosaminidase (units of activity)	**1.55 ± 0.07**	**0.6 ± 0.03**	**0.18 ± 0.08**

Table 6.2. Comparison of means and standard errors of the mean for mediator content of rOv.IL-3-dependent BMMC (IL-3 BMMC; day 19 of culture), conditioned medium-derived BMMC (CM-BMMC; days 21 to 35 of culture) and isolated MMC from parasitized abomasal mucosa (abomasal MMC). (* Data derived from Huntley et al 1992).

CHAPTER 7

THE FUNCTIONAL HETEROGENEITY OF rOv.IL-3 BMMC : THEIR RESPONSE TO SUBSTANCE P, COMPOUND 48/80, CALCIUM IONOPHORE A23187, SMCP AND HEAT-INACTIVATED SMCP.

7.1. Introduction

Comparison of the differential response of mast cell populations to chemical secretagogues (Foreman 1993) is one of the standard methods of defining mast cell heterogeneity (Barrett and Pearce 1993). As discussed in the general introduction, 48/80 and sP are generally activators of the CTMC subset (with some exceptions), whereas A23187 is a non-specific activator of both CTMC and MMC phenotypes (1.5). Mast cell populations generated *in vitro* have also been characterized by comparing their secretagogue activity to that observed *in vivo*. For example, in one study an *in vitro* murine IL-3 BMMC population was considered to share properties with those of murine peritoneal mast cells (Chiu and Burrall 1990). Additionally, chymase, as outlined earlier, can activate rat serosal mast cells (Schick and Austen 1986) and rat CM-BMMC (Gardner 1990) *in vitro*.

Having shown that these agents possess putative secretagogue activity for the ovine dermal mast cell population (Chapters 4 and 5), their application *in vitro* may permit further characterization of rOv.IL-3 BMMC (Chapter 6). The studies described in this Chapter were carried out in order to bring additional criteria to the assessment of ovine mast cell heterogeneity.

7.2. Experiment 7.1 - Mediator Release Studies On rOv.IL-3 BMMC Induced By Calcium Ionophore A23187, Compound 48/80, Substance P, SMCP And Heat-Inactivated SMCP

7.2.1. Experimental Aim And Design

The aim was to determine if rOv.IL-3 BMMC are activated *in vitro* by A23187, sP, 48/80 and SMCP. The release of the granule-associated mediators arylsulfatase and β-hexosaminidase was quantified for all of the secretagogues. Additionally, the release of SMCP was determined for A23187, sP and 48/80.

Bone marrow cells were obtained as described (2.3.1) and grown in the presence of an optimal concentration of rOv.IL-3 (initially 10^{-1} rOv.IL-3 prior to using new, batch-tested CHO cell-derived supernatants). Cultures containing 40-50% mast cells were harvested on days 12 to 16 (Chapter 6) and mediator release studies performed (2.3.15). Where possible, the relative amount of each secretagogue and of the respective diluent in control eppendorfs was the same as that used *in vivo* (Chapters 4 and 5). (As the rOv.IL-3 BMMC were finally suspended in 200µl of medium for the release study, this necessitated increasing the quantity of secretagogue from that used *in vivo* by a factor of four). Thus, A23187 was used at final concentrations of 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M, 48/80 at 250µg/ml and substance P at 10^{-3} M and 10^{-4} M. SMCP and the diluent control was prepared (2.5.1.2), aliquots of each being subjected to heat inactivation (HI) at 64 °C for 10 minutes (2.5.1.3), this resulting in SMCP with a residual activity of about 2% (2.5.1.3). 3.6µg (i.e. 72µg/ml as the final concentration in the eppendorf), 360ng and 36ng SMCP (amounts in µg are per 50µl Earle's medium; 200µl Earle's medium being used per eppendorf, containing 0.5 or 1 x 10^6 cells) and of HI-SMCP and of diluent and HI-diluent were used. Samples were performed in duplicate and, for each agent, measurements were undertaken in two separate rOv.IL-3 BMMC cultures. Assays of SMCP, arylsulfatase and β-hexosaminidase were performed on pellet supernatants following activation as described (2.3.12, 2.3.13, 2.3.14). The release of mediator was expressed as a percentage of the total content or activity of mediator assayed after extraction from separate duplicate, non-stimulated, control cell pellets (2.3.15).

7.2.2. Results

In mediator release studies undertaken with sP, the values for release due to 10^{-3} M sP were appreciably higher in comparison to diluent for all three mediators in each of the experiments (Table 7.1). Diluent-induced β-hexosaminidase release values were similar between the two

experiments (12% and 14.5%), 10^{-3} M sP evoking approximately two to three times this baseline release (Table 7.1). The release of arylsulfatase and SMCP was more variable between experiments and in the diluent values. From these results, sP appears to evoke mediator release from rOv.IL-3 BMMC.

The release of β-hexosaminidase in response to 48/80 was again consistently greater (35.6% and 15%) than in response to diluent alone (3.1% and 8.2%). The baseline values for arylsulfatase again varied between experiments and, for SMCP, were high (41.7% and 46.4%) but there was, again, an apparently specific release of all three mediators in response to challenge (Table 7.2).

For A23187 the values for the diluent controls varied between experiments (Table 7.3). However, the trend was for the higher concentrations of A23187 (10^{-4} M and 10^{-5} M) to evoke an approximate two-fold increase in specific release of mediator (Table 7.3). This would suggest, therefore, that A23187 is a secretagogue for rOv.IL-3 BMMC.

Exposure of rOv.IL-3 BMMC to exogenous SMCP and HI-SMCP failed to increase the release of arylsulfatase or β-hexosaminidase above that observed with diluent alone (Table 7.4). When the concentration of diluent was equivalent to that used for 3.6μg SMCP/50μl medium (Table 7.4), there was 65.3% to 68.9% release of β-hexosaminidase. This is much higher than for other diluents (e.g. sP 12.0% to 14.5%; 48/80, 3.1% to 8.2%; A23187, 4.7% to 25.8%). There is, therefore, no evidence that SMCP or HI-SMCP can evoke mediator release from rOv.IL-3 BMMC.

	Percentage mediator release induced by Substance P		
β-hexosaminidase release	**10^{-3} M sP**	**10^{-4} M sP**	**Diluent**
Experiment 1	36.7%	24.5%	12.0%
Experiment 2	27.5%	21.3%	14.5%
Arylsulfatase release	**10^{-3} M sP**	**10^{-4} M sP**	**Diluent**
Experiment 1	63.0%	31.6%	30.4%
Experiment 2	50.3%	30.1%	11.3%
SMCP release	**10^{-3} M sP**	**10^{-4} M sP**	**Diluent**
Experiment 1	16.7%	5.5%	5.8%
Experiment 2	67.2%	60.8%	46.1%

Table 7.1. The release of the granule constituents arylsulfatase, β-hexosaminidase and SMCP from rOv.IL-3 BMMC in response to stimulation with substance P. The release from aliquots of either 0.5 or 1 x 10^6 cells are expressed as the percentage of the total extracted from a similar aliquot of cells with 2M KCl in 0.02M Tris/HCl pH 7.5 and repeated freeze thawing (Huntley 1991). Each dose of secretagogue or respective diluent control was tested in duplicate.

	Percentage mediator release	
β-hexosaminidase release	**48/80**	**Diluent**
Experiment 1	35.6%	8.2%
Experiment 2	15.0%	3.1%
Arylsulfatase release	**48/80**	**Diluent**
Experiment 1	25.8%	0%
Experiment 2	41.0%	19.6%
SMCP release	**48/80**	**Diluent**
Experiment 1	78.6%	46.4%
Experiment 2	74.0%	41.7%

Table 7.2. The release of the granule constituents arylsulfatase, β-hexosaminidase and SMCP from rOv.IL-3 BMMC in response to stimulation with 250μg/ml compound 48/80. The release from aliquots of either 0.5 or 1 x 10^6 cells are expressed as the percentage of the total extracted from a similar aliquot of cells with 2M KCl in 0.02M Tris/HCl pH 7.5 and repeated freeze thawing (Huntley 1991). Each secretagogue or diluent control sample was tested in duplicate.

	Percentage mediator release induced by A23187				
β-hexosaminidase release	**10^{-4} M**	**10^{-5} M**	**10^{-6} M**	**10^{-7} M**	**Highest diluent release**
Experiment 1	49.2%	54.7%	13.7%	21.2%	25.8%
Experiment 2	23.5%	17.8%	6.6%	6.4%	4.7%
Arylsulfatase release					
Experiment 1	38.4%	24.8%	16.4%	23.4%	19.0%
Experiment 2	70.1%	72.1%	10.1%	35.9%	33.6%
SMCP release					
Experiment 1	21.7%	27.3%	15.1%	12.7%	11.2%
Experiment 2	43.8%	38.6%	33.3%	21.1%	18.8%

Table 7.3. The release of the granule constituents arylsulfatase, β-hexosaminidase and SMCP from rOv.IL-3 BMMC in response to stimulation with A23187. The release from aliquots of either 0.5 or 1 x 10^6 cells are expressed as the percentage of the total extracted from a similar aliquot of cells with 2M KCl in 0.02M Tris/HCl pH 7.5 and repeated freeze thawing (Huntley 1991). Each secretagogue or diluent control sample was tested in duplicate.

	Percentage mediator release		
β-hexosaminidase release	**SMCP**	**Heat-inactivated SMCP**	**Diluent**
Experiment 1			
3.6μg	52.0%	54.1%	68.9%
360ng	15.8%	8.8%	5.8%
36ng	6.4%	9.0%	6.1%
Experiment 2			
3.6μg	66.9%	38.7%	65.3%
360ng	8.9%	11.2%	19.6%
36ng	5.4%	6.2%	8.8%
Arylsulfatase release			
Experiment 1			
3.6μg	32.2%	33.2%	36.6%
360ng	3.3%	16.4%	0%
36ng	0%	0%	0%
Experiment 2			
3.6μg	20.1%	19.8%	25.0%
360ng	19.8%	0%	12.6%
36ng	25.0%	0%	0%

Table 7.4. The release of the granule constituents arylsulfatase, β-hexosaminidase and SMCP from rOv.IL-3 BMMC in response to stimulation with SMCP or HI-SMCP. The release from aliquots of either 0.5 or 1 x 10^6 cells are expressed as the percentage of the total extracted from a similar aliquot of cells with 2M KCl in 0.02M Tris/HCl pH 7.5 and repeated freeze thawing (Huntley 1991). Each dose of secretagogue or respective diluent control sample was tested in duplicate.

7.3. Discussion

Bearing in mind the problems regarding interpretation of the data (see below) and the need for further experimentation to allow statistical analysis (insufficient time was available to repeat these experiments, the series depicted taking five months to complete), some conclusions can be tentatively inferred. Secretagogue activity on rOv.IL-3 BMMC was consistently demonstrable with sP, 48/80 and A23187, but not with SMCP or HI-SMCP. This activity was apparently specific, as SMCP release was a consistent finding. Given that 48/80 does not activate MMC (Table 1.3(b)), it implies that rOv.IL-3 BMMC share characteristics with cells of the CTMC phenotype. (N.B. Substance P has been shown to activate rat intestinal mast cells; Befus et al 1986, Table 1.4). The peptidergic pathway of mast cell activation (through which 48/80 is proposed to act) is stated to be present in human cutaneous mast cells (CTMC phenotype; Mousli et al 1994), which would support the above finding. However, reactivity to 48/80 is not demonstrated by all cutaneous mast cell populations (e.g. bovine; Hunt et al 1991). Given also that rOv.IL-3 BMMC possess some MMC-like properties (Chapter 6), then the cultured cell population may be of mixed phenotype, which would be consistent with the apparent phenotypic heterogeneity of ovine CM-BMMC (Huntley et al 1992) and similar to murine BMMC which may also be heterogeneous (Newlands et al 1991).

There were both similarities and discrepancies between the *in vitro* and *in vivo* effects of the various agents. Although a dose-dependent effect of A23187 on the immediate weal response was observed *in vivo* (Figure 4.2(c), the *in vitro* results do not consistently support a similar effect (Table 7.3). In cultures of ovine CM-BMMC a typical bell-shaped curve of mediator release occurred across the range of 10^{-5} M to 10^{-9} M A23187, with 10^{-6} M A23187 being optimal (Huntley 1991). Interestingly, with the exception of arylsulfatase, the range of values for A23187 diluent-corrected percentage mediator release for rOv.IL-3 BMMC are similar to

those of CM-BMMC (**rOv.IL-3 BMMC** [derived from Table 7.3]: SMCP 1.5% to 25%, β-hexosaminidase 0% to 23.4%, arylsulfatase 0% to 36.5%; **CM-BMMC**: SMCP 0% to 47.6%, β-hexosaminidase 0% to 31.5%, arylsulfatase 0% to 92.5% [Huntley 1991]). Thus, despite the relatively high diluent background levels, specific mediator release by rOv.IL-3 BMMC was demonstrable. The diluents also promoted cutaneous responses (Chapter 4), so further investigation of more suitable diluents for these agents is warranted. Care must be taken when comparing the responses of mast cell populations from different sources (e.g. *in vivo* and *in vitro*) to secretagogues as the relative degree of maturation of these two cell populations may differ markedly, thereby affecting the observed response.

Compound 48/80 at a concentration of 250μg/ml apparently activated mast cells *in vitro* (Table 7.2), but failed to evoke an immediate weal response *in vivo* (Fig. 4.3(c) and Fig. 4.3(d)), despite histological evidence of mast cell degranulation as early as 15 minutes after injection (Fig. 4.5(d)). Substance P needs to be used across a wider range of concentrations *in vitro*, to determine if increased release would be observed with 10^{-5} M sP, consistent with the "window" effect seen *in vivo* (Figure 4.2(a)). Interestingly, there was evidence that 10^{-4} M sP evoked mediator release *in vitro* (Table 7.1), despite failing to evoke a significant weal response *in vivo*. However, optimal concentrations of secretagogues can only readily be maintained *in vitro*, whereas, *in vivo*, it may be rapidly dispersed or even bound in the tissues. Based on the above results (Table 7.4), SMCP and HI-SMCP had no obvious secretagogue activity. Different chymases, like all enzymes, are best compared in terms of their catalytic activities, but it is interesting to note that the concentration of chymase which activated rat serosal mast cells was 50μg/ml (Schick and Austen, 1986) as compared with the concentration of SMCP at 72μg/ml. Thus, although this concentration evoked an immediate cutaneous response accompanied by histological evidence of mast cell degranulation *in vivo*

(Fig.5.1(a), Fig 5.1(b), Fig 5.1(c), Fig. 5.6(a) and Fig. 5.6(b)) it may not have been sufficient to activate rOv.IL-3 BMMC *in vitro* and further dose-response studies will be required. Theoretically, since both sP and 48/80 appeared to evoke mediator release, if SMCP (as a basic cationic protein) was working through the same peptidergic pathway (Mousli et al 1994), then mediator release would have been anticipated. Given that cutaneous mast cells are degranulated as part of the cutaneous response to intradermal injection of SMCP, it can be speculated that rOv.IL-3 BMMC lack this same mechanism of activation, or alternatively that ovine cutaneous mast cells could be activated by inflammatory mediators released through the action of SMCP on native substrates. The possibility of a direct effect of SMCP on vascular endothelium in the *in vivo* response has already been alluded to (Chapter 5).

Inconsistent results were obtained when the medium used in the original release study on ovine CM-BMMC (Tyrodes plus 0.1% gelatin; Huntley et al 1992) was initially used for rOv.IL-3 BMMC. Earle's medium was a satisfactory replacement, there being no discernible benefit of the addition of 0.1% gelatin. With this exception, the protocol for mediator release was as for the original CM-BMMC study (Huntley et al 1992).

A consistent feature in the results outlined above is the relatively high levels of mediator release apparently due to the effect of diluent alone. This may indicate an actual effect of the respective diluents or it could reflect a qualitative difference in rOv.IL-3 BMMC between cultures, with BMMC from certain cultures being more "fragile" and therefore prone to spontaneous mediator release. Under the experimental conditions outlined, this effect would be interpreted as attributable to the diluent. An alternative explanation would be that the apparent spontaneous release could be attributed to cell death. Had time permitted, measurement of the release of a putative marker for cell death for rOv.IL-3 BMMC (e.g. β-glucuronidase) would have been undertaken. The hypothesis of qualitative differences in rOv.IL-3 BMMC between cultures would be supported by the contention that where there are

marked differences in diluent-associated mediator release (e.g. A23187 diluent β-hexosaminidase release; Experiment 1: 25.8%, Experiment 2: 4.7%), there are proportionate differences in secretagogue-induced mediator release (Experiment 1 [10^{-4} M]: 49.2%, Experiment 2 [10^{-4} M]: 23.5%). This would also account for the apparent variability in values for secretagogue-induced mediator release. However, based on the results presented, the possibilities that this is a direct effect of diluent or, alternatively, attributable to cell death cannot be discounted.

One other problem is that the release studies have been undertaken on a non-homogeneous cell population that contains only 40-50% BMMC. Given that results from Chapter 6 suggested that SMCP may be a more reliable indicator of rOv.IL-3 BMMC activation than arylsulfatase or β-hexosaminidase, the possibility that these latter mediators are being produced by other cell types cannot be eliminated. However, 48/80, sP and A23187 all provoked specific release of SMCP.

In order to explicitly define ovine mast cell functional heterogeneity, and to confirm that the sheep holds to the pattern of secretagogue activation seen in other species (Table 1.3(a), Table 1.4, Table 1.5), both dispersed cutaneous and intestinal mast cells should be challenged with sP and 48/80. Alternatively, as planned (Chapter 8), mast cell mediators could be measured in afferent lymph immediately after intradermal challenge with secretagogues. Either method would help confirm that sP and 48/80 are ovine dermal mast cell secretagogues (Chapter 4).

These experiments have established that rOv.IL-3 BMMC are responsive to sP, 48/80 and A23187, but not apparently to SMCP. This contrasts with ovine dermal mast cells, which appear to be activated by all four secretagogues (Chapters 4 and 5). Phenotypically, rOv.IL-3 BMMC possess both MMC-like (SMCP-containing) and CTMC-like (activation by 48/80) characteristics. However, rOv.IL-3 BMMC cannot be considered as a suitable *in vitro*

analogue of the ovine dermal mast cell, as the latter are overwhelmingly SMCP-negative. Therefore, further *in vitro* studies are necessary to generate an ovine BMMC population with a chiefly CTMC phenotype.

CHAPTER 8

GENERAL DISCUSSION

The hypothesis that mast cells participate in cutaneous inflammatory responses is based upon an expanding body of evidence. For example, certain clinical dermatological disorders are associated with mast cell hyperplasia (Yamamoto et al 1995), altered levels of IgE (Halliwell and Longino 1985) or with immediate weal responses (Hawk et al 1980). Additionally, various mast cell-derived mediators (including arachidonic acid metabolites, proteinases and cytokines) can evoke vascular or cellular responses when injected into skin *in vivo* (Colditz 1991b, Colditz and Watson 1992, Walls et al 1994). Finally, and most elegantly, the mast cell-dependency of certain cutaneous responses has been explicitly defined by use of mast cell-deficient or locally mast cell-reconstituted W/W^v mice (reviewed in Galli, Tsai and Wershil 1993). The main focus of these studies has been the contribution of the mast cell-derived chymase, SMCP, to mast cell biology of the sheep. The demonstration that ovine mast cells are heterogeneous with regard to their proteinase content is consistent with findings in other species and leads to a number of questions. For example, only a small proportion of ovine dermal mast cells contain SMCP (recently confirmed with a monoclonal anti-SMCP antibody; J.F.Huntley, *personal communication*) and yet the intradermal injection of SMCP evokes a marked cutaneous response. What, therefore, is the proteinase content of the vast majority of ovine dermal mast cells? Although there is evidence for an additional putative serine endopeptidase in ovine CM-BMMC (Huntley et al 1992), it is not known if this exists in ovine dermal mast cells. Additionally, an ovine tryptase has as yet not been isolated, although SMCP itself possesses "trypsin-like" activity (Pemberton, Huntley and Miller, *submitted*). Given that tryptase can evoke marked cutaneous responses *in vivo* (Walls et al 1994), from a functional standpoint the determination of the presence or absence of tryptase in ovine dermal mast cells is a major priority. This would also expand knowledge of ovine mast cell heterogeneity and allow determination of whether chymase (as in rats and mice) or tryptase (as in the dog and man) is the more abundant ovine proteinase. This is of relevance

also to pulmonary inflammatory responses and, since the sheep has been advocated as a model for human asthma (Clark et al 1995) and tryptase is the predominant enzyme in human mast cells (Walls 1995), the contribution of SMCP relative to tryptase may be of functional significance. The hypothesis that an undetected ovine mast cell tryptase does exist also allows speculation regarding an alternative mechanism for the marked neutrophilic dermal infiltrate that occurs following intradermal injection of SMCP (via hypothetical SMCP-induced mast cell activation), particularly as neutrophilic infiltrates have not been reported in tissues where high levels of SMCP release have been detected (e.g. gastrointestinal tract, discussed below). The demonstration that a small percentage of cutaneous mast cells contained SMCP did, however, validate its use in intradermal challenge studies, this being one of the first comprehensive studies where a native proteinase was intradermally injected into the homologous host. Consistent with studies which have used other chymase preparations *in vivo* (Fräki 1977, Walls et al 1994), a prominent neutrophilic infiltrate was detected after the intradermal injection of SMCP. SMCP evoked a potent weal response (peaking 180 minutes after injection) and a neutrophil influx (which was still increasing from 180 to 360 minutes after injection). Indeed, the highest concentration of 36μg (≈1.3μM) SMCP was not used in time course studies because of the severity of response. This apparent potency can be explained in various ways. Like human chymase (Schechter et al 1989), SMCP is inhibited relatively slowly (≈15 minutes) by its homologous α_1-proteinase inhibitor ($k_{ass} \approx 1.1 \times 10^3$ $M^{-1}s^{-1}$; Pemberton, Huntley and Miller, *submitted*), and, in the absence of any other inhibitor (apart from α_2-macroglobulin), SMCP may be active at some distance from its point of release (or injection). This could, in part, explain the marked and prolonged cutaneous response. Furthermore, the potency of SMCP may be altered by the presence of heparin because the activity of human chymase at pH 7.5 (optimal for interstitial tissues) has recently been shown to be enhanced by the presence of heparin (M^{c}Euen, Sharma and

Walls, 1995). Also, the intradermal injection of human tryptase in conjunction with heparin evokes an immediate cutaneous response in ovine skin that is of greater magnitude than that evoked by tryptase alone (Molinari et al *in press*). Therefore, if the activity of SMCP was similarly up regulated by heparin, the *in vivo* potency of SMCP, when released from mast cells, may have been underestimated in present studies. However, lack of time precluded the co-injection of SMCP with heparin in these studies.

The timing of the development of the SMCP-evoked dermal neutrophilic infiltrate is similar to the timing of ELAM-1 expression that occurs in human skin following mast cell activation (Klein et al 1989). Indeed, mast cell degranulation occurred within 15 minutes of injecting SMCP, and the contribution of histamine to the vascular response needs to be elucidated. Therefore, as discussed in Chapter 5, H_1- and H_2-receptor antagonists should be used in combination with SMCP in cutaneous challenge studies *in vivo* to assess this contribution (Rubenstein et al 1990, Walls et al 1993). More importantly, the question of whether SMCP would potentiate a histamine-induced weal response in the sheep (as with chymase in the dog, Rubenstein et al 1990) needs to be determined. If so, this potentiation by SMCP of a histamine-induced vascular response would provide a further possible explanation for the exaggerated cutaneous weal response. One apparent contradiction from these results is the presence of a marked dermal neutrophilic infiltrate in response to relatively low concentrations of SMCP whereas, in the gut, it is known that relatively high levels of SMCP are released into local lymph and blood during the expulsion of nematodes in immune sheep (Huntley et al 1987) in the absence of a neutrophilic infiltrate (indeed neutrophils are not a feature of worm expulsion in most species; Miller 1993b). One simplistic hypothesis for this contradiction would be that injected SMCP activates dermal mast cells (the majority of which lack SMCP) to release additional proteinases which are absent or in low quantities in the overwhelmingly SMCP-positive mast cells of the gastrointestinal tract, and which recruit neutrophils into the

dermis. Alternatively, there may be differences in the microenvironment of the skin and the gastrointestinal tract. For instance, the substrates for SMCP may differ at the two sites, and peptides released in skin may be chemotactic for neutrophils. The native substrates of SMCP should, therefore, be characterized. Another possibility, since human chymase can generate active IL-1 from its precursor by proteolysis (Kupper et al 1990, Mizutani et al 1991) and, since IL-1 evokes a neutrophilic infiltrate in ovine skin *in vivo* (Colditz and Watson 1992), is that SMCP activates a latent cytokine. Whether such a mechanism exists in ovine skin is unknown. Also, given that bullous lesions were identified on two occasions in SMCP-treated skin, one possible substrate might be basement membrane proteins. Immunohistochemical or immunofluorescent studies could therefore be undertaken to identify the localization of SMCP following intradermal injection, as with human chymase (Sayama et al 1987).

The relative concentrations of proteinase inhibitors at the two sites may be very important. For example, the local lymph draining from the abomasum in immune animals during nematode expulsion contains inhibitors (Huntley et al 1987). If the level of inhibitors was lower in skin than in gut, then SMCP (which is only present in low concentrations in skin) would be relatively more potent in skin. Further investigations should therefore address the nature of the inhibitors of SMCP present in ovine skin. This could be achieved fairly readily by collecting afferent lymph.

Speculatively, SMCP may be able to activate vascular endothelium (or other cell types) directly via protease-activated receptors (Coughlin 1994, Altieri 1995). Activated endothelium can also generate pro-inflammatory cytokines (e.g. IL-8, Schall and Bacon 1994), and could contribute to an exaggerated cutaneous response, in contrast to that evoked by secretagogue-challenge alone.

Impetus has also been provided for investigation of the possible interaction of SMCP and sP in neurogenic inflammation. The cutaneous response to sP injection is accompanied by mast cell degranulation. Given that SMCP cleaves sP *in vitro* (Pemberton, Huntley and Miller *submitted*), then a modulatory role for SMCP in cutaneous responses *in vivo* can be envisaged. SP released from nerve endings could activate dermal mast cells, thereby resulting in mast cell mediator release (including proteinases). Cleavage of sP by proteinases would remove the initiating stimulus and thus regulate the response. This could be investigated by injecting affinity purified anti-SMCP antibodies intradermally prior to the injection of sP, and determining if this prolongs the sP-induced cutaneous response. It is of note that the number of neutrophils in sP-treated sections fell between 180 and 360 minutes following challenge, which is at variance with the other agents tested. Whether this curtailment in response is mediated by an enzymatic effect of SMCP or of another proteinase from dermal mast cells on sP is speculative, but warrants further investigation. This could, for example, be achieved by injecting proteinase inhibitors.

A number of questions raised by these studies and discussed above could have been answered (as originally planned) by further intradermal injections of SMCP and by measuring mediators entering afferent lymph (Smith, M^{c}Intosh and Morris 1970, Hecker 1974). This would have allowed the monitoring of mast cell mediator release (e.g. histamine and possibly SMCP) into afferent lymph immediately following cutaneous challenge, thereby allowing more accurate assessment (in comparison to histological methods) of the effects of putative secretagogues. Additionally, changes in lymph flow, cell output and phenotypic alterations in cell types trafficking into skin following mast cell activation can be measured (Yirrell et al 1991). Also, as in previous studies, the appearance of cytokine activity in afferent lymph draining from the challenge site can be assessed (Haig et al 1992). This would have provided the ideal system in the homologous host for dissecting the cutaneous response to intradermal SMCP-challenge.

Although prefemoral afferent lymphatics in seven sheep were successfully cannulated, lymph flow stopped in all subjects within three days of cannulation. Therefore, further investigation of the mechanism of action of SMCP in ovine skin and of the effects of direct mast cell activation (using the secretagogues identified previously *in vivo*) on altering inflammatory cell trafficking through the dermis could not be undertaken. One can speculate that dermal mast cells, if they are directly activated by SMCP, immediately release TNF-α (Gordon and Galli 1990a) which, in turn, activates vascular endothelium and upregulates the expression of adhesion molecules, particularly ELAM-1 (Klein et al 1989), thereby initiating the neutrophilic infiltrate typically associated with the response to TNF-α in ovine skin (Colditz and Watson 1992). The activation of endothelial cells and other cell types by TNF-α would subsequently result in the expression of IL-8 and thus augment neutrophil recruitment in ovine skin (Seow et al 1994). In such an acute response, the expression of ELAM-1 would be maximal within 4 to 6 hours, and have subsided by 24 hours (Cotran 1987), consistent with the observed decline in cellular infiltrate from 24 to 72 hours following challenge. Obviously, other cytokines from a variety of cell types will probably also be involved in both the initiation (IL-1, IL-6, chemokines) and resolution (IL-3, IL-13, TGF-β) of the inflammatory response. The cutaneous vascular response to mast cell-derived histamine is likely to include the rapid appearance of histamine and other mast cell-derived mediators in afferent lymph, and an increased lymph flow rate from a basal 1.5 to 3.0 ml/hr (containing 75-85% lymphocytes and 500 to 800 cells/mm^3; Smith, M^{c}Intosh and Morris 1970) peaking 3 hours after injection, the increased lymph flow subsequently subsiding in association with the declining weal response. The nature of the likely cellular response is not known but the types of cell present and their state of activation both require investigation. Had time allowed, one alternative to lymphatic cannulation that could have been attempted was to employ microdialysis techniques, where mediators (e.g. histamine) can be measured in the dialysate draining from activated skin into

implanted dermal microdialysis fibres following injection of secretagogues (Petersen et al 1994).

The apparent responsiveness of ovine dermal mast cells to sP and 48/80 suggest that the functional phenotype of these cells is consistent with that of the CTMC subtype in other species, although this point was to have been explored further by the cannulation studies described above. The kinetics of growth and the mediator content of rOv.IL-3 BMMC have been characterized (Chapter 6), but the conclusions regarding their responses to secretagogues remains tentative (Chapter 7). Because they contain SMCP, rOv.IL-3 BMMC are MMC-like (in comparison to *in vivo* results from ovine skin and gastrointestinal tract) but also have a CTMC-like characteristic, in view of their response to sP and 48/80. (This is with the proviso that, as exceptions to the general rule, bovine cutaneous mast cells do not respond to 48/80 (Table 1.3(b); Hunt et al 1991) and rat intestinal mast cells have been shown to respond to sP (Table 1.4; Befus et al 1986). It is apparent that IL-3 is not the sole factor required for ovine BMMC development, particularly as ovine CM-BMMC appear structurally more mature and survive longer *in vitro* (Huntley et al 1992). Indeed, preliminary studies indicate that rOv.IL-3 BMMC grown in the presence of recombinant ovine SCF are more developed than comparable rOv.IL-3 BMMC (J.F.Huntley, personal communication).

Immunohistochemical detection of granule proteinases in rOv.IL-3 BMMC is currently limited to SMCP. Thus, it is not known if rOv.IL-3 BMMC represent a homogenous population with respect to proteinase expression (as in rat BMMC; Haig et al 1988a) or whether it is heterogeneous (as in mouse BMMC; Newlands et al 1991). As rOv.IL-3 BMMC contain low quantities of SMCP, then this would suggest similarities with mouse, rather than rat, BMMC populations based on proteinase content. Again, the identification of further ovine mast cell proteinases would be of importance, as this would greatly help with the further delineation of

ovine mast cell heterogeneity both *in vivo* and *in vitro*. For example, these would help to detect phenotypic changes in proteinase content that may occur in rOv.IL-3 BMMC due to SCF addition *in vitro*.

In summary, these studies have greatly expanded our knowledge of the ovine mast cell and have defined mast cell heterogeneity in this species. Furthermore, a participating role for mast cells and, importantly, for SMCP in ovine cutaneous inflammatory responses has been elucidated.

BIBLIOGRAPHY

Abe, T. and Nawa, Y., 1988. Worm expulsion and mucosal mast cell response induced by repetitive IL-3 administration in *Strongyloides ratti*-infected nude mice. Immunology, **63**: 181-185.

Agis, H. and Valent, P., 1995. Molecules involved in the development of human basophils and mast cells. In: Y. Kitamura, S. Yamamoto, S.J. Galli and M.W. Greaves (Editors), Biological and Molecular Aspects of Mast Cell and Basophil Differentiation and Function. Raven Press Ltd., New York, pp. 119-130.

Alabadie, M.S.K., Senior, H.J., Bleehen, S.S. and Gawkrodger, D.J., 1994. Neuropeptides and neuronal marker in atopic-dermatitis - a quantified immunohistochemical study. European Journal of Dermatology, **4**: 394-398.

Alam, R., Kumar, D., Anderson-Walters, D. and Forsyth, P.A., 1994. Macrophage inflammatory protein-1α and monocyte chemoattractant peptide-1 elicit immediate and late cutaneous reactions and activate murine mast cells in vivo. Journal of Immunology, **152**: 1298-1303.

Aldenborg, F. and Enerbäck, L., 1988. Histochemical heterogeneity of dermal mast cells in athymic and normal rats. Histochemical Journal, **20**: 19-28.

Aldenborg, F. and Enerbäck, L., 1994. The immunohistochemical demonstration of chymase and tryptase in human intestinal mast cells. Histochemical Journal, **26**: 587-596.

Alter, S.C., Metcalfe, D.D., Bradford, T.R. and Schwartz, L.B., 1987. Regulation of human mast cell tryptase. Effects of enzyme concentration, ionic strength and the structure and negative charge density of polysaccharides. Biochemical Journal, **248**: 821-827.

Alter, S.C. and Schwartz, L.B., 1989. Effect of histamine and divalent cations on the activity and stability of tryptase from human mast cells. Biochemica et Biophysica Acta, **991**: 426-430.

Alter, S.C., Kramps, J.A., Janoff, A. and Schwartz, L.B., 1990. Interactions of human mast cell tryptase with biological protease inhibitors. Archives of Biochemistry and Biophysics, **276**: 26-31.

Altieri, D.C., 1995. Proteases and protease receptors in modulation of leukocyte effector functions. Journal of Leukocyte Biology, **58**: 120-127.

Amon, U., Nitshcke, M., Dieckmann, D., Gibbs, B.F., Wehrhahn, C. and Wolff, H.H., 1994. Activation and inhibition of mediator release from skin mast cells: a review of *in vitro* experiments. Clinical and Experimental Allergy, **24**: 1098-1104.

Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D. and Williams, D.E., 1990. Molecular cloning of a mast cell growth factor, a haematopoietin that is active in both membrane-bound and soluble forms. Cell, **63**: 235-243.

Anderson, J.A., Miller, F.N., Sims, D.E. and Edwards, M.J., 1994. Tumor necrosis factor causes microvascular protein leakage independently of neutrophils or mast cells. Journal of Surgical Research, **56**: 485-490.

Arai, K., Lee, K., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T., 1990. Cytokines: coordinators of immune and inflammatory responses. Annual Reviews of Biochemistry, **59**: 783-836.

Aschner, J.L., Lennon, J.M., Fenton, J.W., Aschner, M. and Malik, A.B., 1990. Enzymatic activity is necessary for thrombin-mediated increase in vascular permeability. American Journal of Physiology, **259**: L270-L273.

Askenase, P.W., 1977. Role of basophils, mast cells and vasoamines in hypersensitivity reactions with a delayed time course. Progress in Allergy, **23**: 199-320.

Banchereau, J., De Paoli, P., Vallé, A., Garcia, E. and Rousset, F., 1991. Long term human B cell lines dependent on interleukin-4 and antibody to CD40. Science, **251**: 70-72.

Baraniuk, J.N., Kowalski, M.L. and Kaliner, M.A., 1990. Neuropeptides in the skin. In: J.D.Bos (Editor), Skin Immune System. CRC Press Inc., Florida, pp. 307-326.

Barrett, K.E., Ali, H. and Pearce, F.L., 1985. Studies on histamine secretion from enzymically dispersed cutaneous mast-cells of the rat. Journal of Investigative Dermatology, **84**: 22-26.

Barrett, K.E. and Pearce, F.L., 1993. Mast cell heterogeneity. In: J.C. Foreman, (Editor), Immunopharmacology of Mast Cells and Basophils. Academic Press Limited, New York, pp. 29-42.

Becker, A.B., Fan Chung, K., M^{c}Donald, D.M., Lazarus, S.C., Frick, O.L. and Gold, W.M., 1985. Mast cell heterogeneity in dog skin. The Anatomical Record, **213**: 477-480.

Befus, D., Goodacre, R., Dyck, N. and Bienenstock, J., 1985. Mast cell heterogeneity in man. I. Histologic studies of the intestine. International Archives of Allergy and Applied Immunology, **76**: 232-236.

Befus, D., Lee, T., Goto, T., Goodacre, R., Shanahan, F. and Bienenstock, J., 1986. Histologic and functional properties of mast cells in rats and humans. In: A.D.Befus, J.Bienenstock and J.A.Denburg (Editors), Mast cell Differentiation and Heterogeneity. Raven Press Ltd., New York, pp. 205-213.

Benditt, E.P. and Arase, M., 1959. An enzyme in mast cells with properties like chymotrypsin. Journal of Experimental Medicine, **110**: 451-460.

Benyon, R.C., Church, M.K., Clegg, L.S. and Holgate, S.T., 1986. Dispersion and characterization of mast cells from human skin. International Archives of Allergy and Applied Immunology, **79**: 332-334.

Benyon, R.C., Lowman, M.A. and Church, M.K., 1987. Human skin mast cells: their dispersion, purification, and secretory characterization. The Journal of Immunology, **138**: 861-867.

Benyon, R.C., Robinson, C., Holgate, S.T. and Church, M.K., 1987. Prostaglandin D_2 release from human skin mast cells in response to ionophore A23187. British Journal of Pharmacology, **92**: 635-638.

Benyon, R.C., Robinson, C., and Church, M.K., 1989. Differential release of histamine and eicosanoids from human skin mast cells activated by IgE-dependent and non-immunological stimuli. British Journal of Pharmacology, **97**: 898-904.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S. and Gimbrone, M.A., 1987. Identification of an inducible endothelial leukocyte adhesion molecule. Proceedings of the National Academy of Sciences of the USA, **84**: 9238-9242.

Bianchine, P.J., Burd, P.R. and Metcalfe, D.D., 1992. IL-3-dependent mast cells attach to plate-bound vitronectin: demonstration of augmented proliferation in response to signals transduced via cell surface vitronectin receptors. Journal of Immunology, **149**: 3665-3671.

Bienenstock, J., (1988) An update on mast cell heterogeneity.
Journal of Allergy and Clinical Immunology, **81**: 763-769.

Bischoff, S.C. and Dahinden, C.A., 1992. c-kit ligand: a unique potentiator of mediator release by human lung mast cells. Journal of Experimental Medicine, **175**: 237-244.

Bos, J.D. and Kapsenberg, M.L., 1986. The skin immune system. Its cellular constituents and their interactions. Immunology Today, **7**: 235-240.

Bostock, D.E., 1973. The prognosis following surgical removal of mastocytomas in dogs. Journal of Small Animal Practice, **14**: 27-40.

Bostock, D.E., 1986. Neoplasms of the skin and subcutaneous tissues in dogs and cats. British Veterinary Journal, **142**: 1-19.

Boswell, H.S., Mochizuki, D.Y., Burgess, G.S., Gillis, S., Walker, E.B., Anderson, D. and Williams, D.E., 1990. A novel mast cell growth factor (MCGF-3) produced by marrow-adherent cells that synergizes with interleukin 3 and interleukin 4.
Experimental Haematology, **18**: 794-800.

Bradding, P., Feather, I.H., Howarth, P.H., Mueller, R., Roberts, J.A., Britten, K., Bews, J.P.A., Hunt, T.C., Okayama, Y., Heusser, C.H., Bullock, G.R., Church, M.K. and Holgate, S.T., 1992. Interleukin 4 is localized to and released by human mast cells.
Journal of Experimental Medicine, **176**: 1381-1386.

Bradding, P., Feather, I.H., Wilson, S., Bardin, P.G., Heusser, C.H., Holgate, S.T. and Howarth, P.H., 1993. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. Journal of Immunology, **151**: 3853-3865.

Bradding, P., Okayama, Y., Howarth, P.H., Church, M.K. and Holgate, S.T., 1995. Heterogeneity of human mast cells based on cytokine content.
Journal of Immunology, **155**: 297-307.

Brain, S.D. and Williams, T.J., 1988. Substance P regulates the vasodilator activity of calcitonin gene-related peptide. Nature, **335**: 73-75.

Briggaman, A.R., Schechter, N.M., Fräki, J.E. and Lazarus, G.S., 1984. Degradation of the epidermal-dermal junction by proteolytic enzymes from human skin and human polymorphonuclear leukocytes. Journal of Experimental Medicine, **160**: 1027-1042.

Brix, H., 1963. Quantitative untersuchungen des mastzellengehalts funktionell unterschiedlicher belasteter normaler menschlicher haut mit kovarianzanalytischer auswertung. Thesis, The University of Berlin.

Broide, D.H., Metcalfe, D.D. and Wasserman, S.I., 1988. Functional and biochemical characterization of rat bone marrow derived mast cells.
Journal of Immunology, **141**: 4298-4305.

Buchanan, M.R., Vazquez, M.J. and Gimbrone, M.A., 1983. Arachidonic acid metabolism and the adhesion of human polymorphonuclear leukocytes to cultured vascular endothelial cells. Blood, **62**: 889-895.

Buckley, M.G., Williams, C.M.M., Thompson, J., Pryor, P., Ray, K., Butterfield, J.H., Coleman, J.W., 1995. IL-4 enhances IL-3 and IL-8 gene expression in a human leukemic mast cell line. Immunology, **84**: 410-415.

Bulman, A.S. and Heyderman, E., 1981. Alkaline phosphatase for immunocytochemical labelling: problems with endogenous enzyme activity.
Journal of Clinical Pathology, **34**: 1349-1351.

Burd, P.R., Rogers, H.W., Gordon, J.R., Martin, C.A., Jayaraman, S., Wilson, S.D., Dvorak, A.M., Galli, S.J. and Dorf, M.E., 1989. Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines.
Journal of Experimental Medicine, **170**: 245-257.

Burd, P.R., Costa, J.J., Metcalfe, D.D. and Siebenlist, U., 1990. Cytokine gene expression occurs via multiple pathways in primary murine bone marrow mast cells.
FASEB Journal, **4**: A1705

Burwen, S.J., 1982. Recycling of mast cells following degranulation *in vitro*: an ultrastructural study. Tissue & Cell, **14**: 125-134.

Camussi, G., Aglietta, M., Malavasi, F., Tetta, C., Piacibello, W., Sanavio, F. and Bussolino, F., 1983. The release of platelet-activating factor from human endothelial cells in culture. Journal of Immunology, **131**: 2397-2403.

Caughey, G.H., Lazarus, S.C., Viro, N.F., Gold, W.M. and Nadel, J.A., 1988a. Tryptase and chymase: comparison of extraction and release in two dog mastocytoma lines.
Immunology, **63**: 339-344.

Caughey, G.H., Viro, N.F., Lazarus, S.C. and Nadel, J.A., 1988b. Purification and characterization of dog mastocytoma chymase: identification of an octapeptide conserved in chymotryptic leukocyte proteinases. Biochemica et Biophysica Acta, **952**: 142-149.

Caughey, G.H., Viro, N.F., Calonico, L.D., M^{c}Donald, D.M., Lazarus, S.C., and Gold, W.M., 1988c. Chymase and tryptase in dog mastocytoma cells: asynchronous expression as revealed by enzyme cytochemical staining.
Journal of Histochemistry and Cytochemistry, **36**: 1053-1060.

Caughey, G.H., Leidig, F., Viro, N.F. and Nadel, J.A., 1988d. Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase.
Journal of Pharmacology and Experimental Therapeutics, **244**: 133-137.

Caughey, G.H., Zerweck, E.H. and Vanderslice, P., 1991. Structure, chromosomal assignment, and deduced amino acid sequence of a human gene for mast cell chymase.
Journal of Biological Chemistry, **266**: 12956-12963.

Champion, R.H. and Burton, J.L., 1992. Diagnosis of skin disease. In: R.H. Champion, J.L. Burton and F.J.G. Ebling (Editors), Rook/Wilkinson/Ebling Textbook of Dermatology 5th Edition, Blackwell Scientific Publications, Oxford, pp. 167-168.

Charlesworth, E.N., 1994. The skin as a model to study the pathogenesis of IgE-mediated acute and late-phase responses. Journal of Allergy and Clinical Immunology, **94**: 1240-1250.

Chen, Z., Irani, A.A., Bradford, T.R., Craig, S.S., Newlands, G., Miller, H., Huff, T., Simmons, W.H., and Schwartz, L.B., 1993. Localization of rat tryptase to a subset of the connective tissue type of mast cell.
Journal of Histochemistry and Cytochemistry, **41**: 961-969.

Chernin, J., Miller, H.R.P., Newlands, G.F.J. and M^{c}Laren, D.J., 1988. Proteinase phenotypes and fixation properties of rat mast cells in parasitic lesions caused by *Mesocestoides corti* - selective and site-specific recruitment of mast cell subsets. Parasite Immunology, **10**: 433-442.

Chiu, H.F. and Burrall, B.A., 1990. Effect of interleukin 3 on the differentiation and histamine content of cultured bone marrow mast cells. Agents and Actions, **31**: 197-203.

Chiu, R. and Lagunoff, D., 1972. Histochemical comparison of vertebrate mast cells. Histochemical Journal, **4**: 135-144.

Church, M.K., Benyon, R.C., Rees, P.H., Lowman, M.A., Campbell, A.M., Robinson, C. and Holgate, S.T., 1989. Functional heterogeneity of human mast cells. In: S.J.Galli and K.F.Austen (Editors), Mast Cell and Basophil Differentiation and Function in Health and Disease. Raven Press Ltd., New York, pp 161-170.

Claman, H.N., 1989. Mast cell changes in a case of rapidly progressive scleroderma - ultrastructural analysis. Journal of Investigative Dermatology, **92**: 290-295.

Clark, J.M., Abraham, W.M., Fishman, C.E., Forteza, R., Ahmed, A., Cortes, A., Warne, R.L., Moore, W.R. and Tanaka, R.D., 1995. Tryptase inhibitors block allergen-induced airway and inflammatory responses in allergic sheep.
American Journal of Respiratory and Critical Care Medicine, **152**: 2076-2083.

Cohan, V.L., Massey, W.A., Gittlen, S.D., Charlesworth, E.N., Warner, J.A., Kagey-Sobotka, A. and Lichtenstein, L.M., 1989. The heterogeneity of human histamine containing cells. In: S.J.Galli and K.F.Austen (Editors), Mast Cell and Basophil Differentiation in Health and Disease. Raven Press, New York, pp. 149-159.

Colditz, I.G., 1988. Two patterns of early neutrophil accumulation in acute inflammatory lesions. Inflammation, **12**: 251-261.

Colditz, I.G., 1991a. Desensitization mechanisms regulating plasma leakage and neutrophil emigration. In: J.L.Gordon (Editor), Vascular Endothelium: Interactions With Circulating Cells. Elsevier, New York, pp. 175-187.

Colditz, I.G., 1991b. The induction of plasma leakage in skin by histamine, bradykinin, activated complement, platelet-activating factor and serotonin.
Immunology and Cell Biology, **69**: 215-219.

Colditz, I.G., 1995. *Personal communication.*

Colditz, I.G. and Movat, H.Z., 1984. Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens.
Journal of Immunology, **133**: 2169-2173.

Colditz, I.G., Zwahlen, R.D. and Baggiolini, M., 1990. Neutrophil accumulation and plasma leakage induced in vivo by neutrophil-activating peptide-1.
Journal of Leukocyte Biology, **48**: 129-137.

Colditz, I.G. and Watson, D.L., 1992. The effect of cytokines and chemotactic agonists on the migration of T lymphocytes into skin. Immunology, **76**: 272-278.

Colditz, I.G., Lax, J., Mortimer, S.I., Clarke, R.A. and Beh, K.J., 1994. Cellular inflammatory responses in skin of sheep selected for resistance or susceptibility to fleece rot and fly strike. Parasite Immunology, **16**: 289-296.

Columbo, M., Horowitz, E.M., Botana, L.M., MacGlasan, D.W., Bochner, B.S., Gillis, S., Zsebo, K.M., Galli,S.J. and Lichtenstein, L.M., 1992. The human recombinant c-kit receptor ligand, rhSCF, induces mediator release from human cutaneous mast cells and enhances IgE-dependent mediator release from both human skin mast cells and peripheral basophils. Journal of Immunology, **149**: 599-608.

Compton, A.S., 1952. A cytochemical and cytological study of the connective tissue mast cell. American Journal of Anatomy, **91**: 301-329.

Connan, R.M. and Lloyd, S., 1988. Seasonal allergic dermatitis in sheep. The Veterinary Record, **123**: 335-337.

Copeland, N.G., Gilbert, D.J., Cho, B.C., Donovan, P.J., Jenkins, N.A., Cosman, D., Anderson, D., Lyman, S.D. and Williams, D.E., 1990. Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell, **63**: 175-183.

Copeman, D.B., 1965. Histamine in canine demodicosis. Thesis, Purdue University, West Lafayette, Indiana.

Cotran, R.S., 1987. New roles for the endothelium in inflammation and immunity. American Journal of Pathology, **129**: 407-413.

Coughlin, S.R., 1994. Protease-activated receptors start a family. Proceedings of the National Academy of Sciences of the USA, **91**: 9200-9202.

Coulson, I.H. and Holden, C.A., 1990. Cutaneous reactions to substance P and histamine in atopic dermatitis. British Journal of Dermatology, **122**: 343-349.

Craig, S.S., Schechter, N.M. and Schwartz, L.B., 1988. Ultrastructural analysis of human T and TC mast cells identified by immunoelectron microscopy. Laboratory Investigation, **58**: 683-691.

Craig, S.C., Irani, A.A., Metcalfe, D.D. and Schwartz, L.B., 1993. Ultrastructural localization of heparin to human mast cells of the MC_{TC} and MC_{T} types by labelling with antithrombin III-gold. Laboratory Investigation, **69**: 552-561.

Crowle, P.K. and Phillips, D.E., 1983. Characteristics of mast cells in Chediak-Higashi mice: light and electron microscopic studies of connective tissue and mucosal mast cells. Experimental Cell Biology, **51**: 130-139.

Cybulsky, M.Y., Colditz, I.G. and Movat, H.Z., 1986. The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. American Journal of Pathology, **124**: 367-362.

Dahlén, S-E., Björk, J., Hedqvist, P., Arfors, K-E., Hammarström, S., Lindgren, J-A. and Samuelsson, B., 1981. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *in vivo* effects with relevance to the acute inflammatory response. Proceedings of the National Academy of Sciences of the USA, **78**: 3887-3891.

Dastych, J., Costa, J.J., Thompson, H.L. and Metcalfe, D.D., 1991. Mast cell adhesion to fibronectin. Immunology, **73**: 478-484.

Dayton, E.T., Ogawa, M., Serafin, W.E., Austen, K.F., Levi Schaffer, F., Stevens, R.L., 1988. 3T3 fibroblasts induce cloned interleukin 3-dependent mouse mast cells to resemble connective tissue mast cells in granular constituency.
Proceedings of the National Academy of Sciences of the USA, **85**: 569-572.

Debuf, Y., 1994. In: Y. Debuf (Editor), The Veterinary Formulary, 2nd Edn.
The Pharmaceutical Press, London, pp. 309-310.

Delara, J.M.T., Okayama, Y., M^{c}Euen, A.R., Heusser, C.H., Church, M.K. and Walls, A.F., 1994a. Release and inactivation of interleukin-4 by mast cells.
Annals of the New York Academy of Sciences, **725**: 50-58.

Delara, J.M.T., M^{c}Euen, A.R., Okayama, Y., Mackie, R.T., Peng, Q., Heusser, C.H., Church, M.K. and Walls, A.F., 1994b. Interleukin-4 degradation by human mast cell chymotryptic proteases. Clinical and Experimental Allergy, **24**: 191.

Del Prete, G.F., De Carli, M., Mastromauro, C., Biagiotti, R., Macchia, D., Falagiani, P., Ricci, M. and Romagnani, S., 1991. Purified protein derivative (PPD) of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) (TES) of *Toxocara canis* select human T cell clones with stable and opposite (Th1 or Th2) profile of cytokine production.
Journal of Clinical Investigation, **88**: 346-350.

Denburg, J.A., Befus, A.D. and Bienenstock, J., 1980. Growth and differentiation *in vitro* of mast cells from mesenteric lymph nodes of *Nippostrongylus brasiliensis*-infected rats.
Immunology, **41**: 195-202.

De Viñals, R.R., 1931. Les "Mastzellen"dans le cancer expèrimental de la souris blanche.
C.R.C. Soc. Biol. (Paris), **108**: 177.

Dowd, P.M., Camp, R.D.R. and Greaves, M.W., 1989. Human recombinant interleukin-1a is proinflammatory in normal human skin. Skin Pharmacology, **1**: 30-37.

Dozza, G. and Rampichini, L., 1963. Caratteristiche istochimiche, numero delle mastzellen per mm^2 di diversi, distretti cutanei de (Equus asinus) E.reattivita pomfoide delle stesse sedi all´ inoculazione intracutanea di 48/80 (istaminoliberatore).
Archivio Veterinario Italiano, **14**: 555-556.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A. and Springer, T.A., 1986. Induction by IL-1 and interferon-γ, tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). Journal of Immunology, **137**: 245-254.

Dvorak, A.M., 1986. Morphologic expressions of maturation and function can affect the ability to identify mast cells and basophils in man, guinea pig and mouse. In: A.D. Befus, J. Bienenstock and J.A. Denburg (Editors), Mast Cell Differentiation and Heterogeneity.
Raven Press, New York, pp. 95-114.

Dvorak, A.M., Mihm, M.C., Dvorak, H.F., 1976. Morphology of delayed-type hypersensitivity reactions in man. II. Ultrastructural alterations affecting the microvasculature and the tissue mast cells. Laboratory Investigation, **34**: 179-191.

Dvorak, A.M., Tepper, R.I., Weller, P.F., Morgan, E.S., Estrella, P., Monahan-Earley, R.A. and Galli, S.J., 1994. Piecemeal degranulation of mast cells in the inflammatory eyelid lesions of interleukin-4 transgenic mice. Evidence of mast cell histamine release in vivo by diamine oxidase-gold enzyme-affinity ultrastructural cytochemistry. Blood, **83**: 3600-3612.

Eady, R.A.J., Cowen, T., Marshall, T.F., Plummer, V. and Greaves, M.W., 1979. Mast cell population density, blood vessel density, and histamine content in normal human skin. British Journal of Dermatology, **100**: 623-633.

Ebertz, J.M., Hirshman, C.A., Kettelkamp, N.S., Uno, H. and Hanifin, J.M., 1987. Substance P induced histamine release in human cutaneous mast cells. Journal of Investigative Dermatology, **88**: 682-685.

Ehrlich, P., 1877. Beiträge zur kenntnis der anilinfärbungen und ihre verwndung in der mikroskopischen technik. Arch. Mikroanat., **13**: 263-277.

Ehrlich, P., 1878. Beiträge zur theorie und praxis der histologischen färbung. Thesis, The University of Leipzig.

Ehrlich, P., 1879. Beiträge zur kenntnis der granulierten bindegewebszellen und der eosinophilen leukozyten. Arch. Anat. Physiol., **3**: 166-169.

Eklund, K.K., Ghildyal, N., Austen, K.F. and Stevens, R.L., 1993. Induction by IL-9 and suppression by IL-3 and IL-4 of the levels of chromosome-14-derived transcripts that encode late-expressed mouse mast cell proteases. Journal of Immunology, **151**: 4266-4273.

Elias, J., Boss, E. and Kaplan, A.P., 1986. Studies of the cellular infiltrate of chronic idiopathic urticaria - prominence of lymphocytes-T, monocytes, and mast cells. Journal of Allergy and Clinical Immunology, **78**: 914-918.

Emerson, J.L. and Cross, R.F., 1965. The distribution of mast cells in normal canine skin. American Journal of Veterinary Research, **26**: 1379-1382.

Enerbäck, L., 1966a. Mast cells in rat gastrointestinal mucosa. I. Effect of fixation. Acta Pathologica, Microbiologica, et Immunologica Scandinavica, **66**: 289-302.

Enerbäck, L., 1966b. Mast cells in rat gastrointestinal mucosa. II. Dye binding and metachromatic properties. Acta Pathologica, Microbiologica, et Immunologica Scandinavica, **66**: 303-312.

Enerbäck, L., 1966c. Mast cells in the rat gastrointestinal mucosa. III. Reactivity towards compound 48/80. Acta Pathologica, Microbiologica, et Immunologica Scandinavica, **66**: 312-322.

Enerbäck, L., 1987. Mucosal mast cells in the rat and man. International Archives of Allergy and Applied Immunology, **82**: 249-253.

Enerbäck, L., Kolset, S.O., Kusche, M., Hjerpe, A. and Lindahl, U., 1985. Glycosaminoglycans in rat mucosal mast cells. Biochemical Journal, **227**: 661-668.

Enerbäck, L., Pipkorn, U., Aldenborg, F. and Wingren, U., 1989. Mast cell heterogeneity in man: properties and function of human mucosal mast cells. In: S.J.Galli and K.F.Austen (Editors), Mast Cell and Basophil differentiation and Function in Health and Disease. Raven Press, New York, pp. 27-37.

Falus, A. and Merétey, K., 1992. Histamine: an early messenger in inflammatory and immune reactions. Immunology today, **13**: 154-156.

Fantini, F., Magnoni, C., Pincelli, C. and Giannetti, A., 1995. Neurogenic inflammation and the skin: neural modulation of cutaneous inflammatory reactions.
European Journal of Dermatology, **5**: 349-357.

Finkelman, F.D., Katona, I.M., Urban, J.J., Snapper, C.M., Ohara, J. and Paul, W.E., 1986. Suppression of in vivo polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1.
Proceedings of the National Academy of Sciences of the USA, **83**: 9675-9678.

Fiorucci, L., Erba, F. and Ascoli, F., 1992. Bovine tryptase: purification and characterization. Biological Chemistry Hoppe-Seyler, **373**: 483-490.

Flanagan. J.G. and Leder, P., 1990. The *kit* ligand: a cell surface molecule altered in steel mutant fibroblasts. Cell, **63**: 185-194.

Flanagan, J.G., Chan, D.C. and Leder, P., 1991. Transmembrane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant.
Cell, **64**: 1025-1035.

Foreman, J.C., 1987. Substance P and calcitonin gene-related peptide : effects on mast cells and in human skin. International Archives of Allergy and Applied Immunology, **82**: 366-371.

Foreman, J.C., 1993. Non-immunological stimuli of mast cells and basophil leucocytes. In: J.C. Foreman, (Editor), Immunopharmacology of Mast Cells and Basophils. Academic Press Limited, New York, pp. 57-69.

Foreman, J.C., Mongar, J.L. and Gomperts, B.D., 1973. Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process.
Nature, **245**: 249-251.

Foreman, J.C. and Mongar, J.L., 1973. The action of lanthunum and manganese on anaphylactic histamine secretion. British Journal of Pharmacology, **48**: 527-537.

Fox, C.C., Kagey-Sobotka, A., Schleimer, R.P., Peters, S.P., MacGlashan, D.W. and Lichtenstein, L.M., 1985. Mediator release from human basophils and mast cells from lung and intestinal mucosa.
International Archives of Allergy and Applied Immunology, **77**:130-136.

Foxwell, B.M.J., Barrett, K. and Feldmann, M., 1992. Cytokine receptors: structure and signal transduction. Clinical and Experimental Immunology, **90**: 161-169.

Fräki, J.E., 1977. Human skin proteases: effect of separated proteases on vascular permeability and leukocyte emigration in the skin.
Acta Dermato-Venereologica (Stockholm), **57**: 393-398.

Franconi, G.M., Graf, P.D., Lazarus, S.C., Nadel, J.A. and Caughey, G.H., 1989. Mast cell tryptase and chymase reverse airway smooth muscle relaxation induced by vasoactive intestinal peptide in the ferret.
Journal of Pharmacology and Experimental Therapeutics, **248**: 947-951.

Füreder, W., Agis, H., Willheim, M., Bankl, H.C., Maier, U., Kishi, K., Müller, M.R., Czerwenka, K., Radaszkiwicz, T., Butterfield, J.H., Klappacher, G.W., Sperr, W.R., Oppermann, M., Lechner, K. and Valent, P., 1995a. Differential expression of complement receptors on human basophils and mast cells. Evidence for mast cell heterogeneity and CD88/C5aR expression on skin mast cells. Journal of Immunology, **155**: 3152-3160.

Füreder, W., Agis, H., Semper, H., Keil, F., Maier, U., Müller, M.R., Czerwenka, K., Höfler, H., Lechner, K. and Valent, P., 1995b. Differential response of human basophils and mast cells to recombinant chemokines. Annals of Hematology, **70**: 251-258.

Furitsu, T., Saito, H., Dvorak, A.M., Schwartz, L.B., Irani, A.M., Burdick, J.F. and Ishizaka, T., 1989. Development of human mast cells *in vitro*.
Proceedings of the National Academy of Sciences of the USA, **86**: 10039-10043.

Galli, S.J., 1990. New insights into the "riddle of the mast cells": microenvironmental regulation of mast cell development and phenotypic heterogeneity.
Laboratory Investigation, **62**: 5-33.

Galli, S.J., 1993. New concepts about the mast cell.
New England Journal of Medicine, **328**: 257-265.

Galli, S.J., Arizono, N., Murakami, T., Dvorak, A.M. and Fox, J.G., 1987. Development of large numbers of mast cells at sites of idiopathic chronic dermatitis in genetically mast cell-deficient $WBB6F_1$-W/W^v mice. Blood, **69**: 1661-1666.

Galli, S.J., Tsai, M. and Wershil, B.K., 1993. The *c-kit* receptor, stem cell factor, and mast cells. W*hat each is teaching us about the others.*
American Journal of Pathology, **142**: 965-974.

Galli, S.J., Iemura, A., Gavlick, D.S., Gamba-Vitalo, C., Zsebo, K.M. and Andrews, R.G., 1993. Reversible expansion of primate mast cell populations in vivo by stem cell factor.
Journal of Clinical Investigation, **91**: 148-152.

Gardner, T.W., 1990. The actions of granule proteases in the activation of rat connective tissue and mucosal mast cells. B.Sc.(Hons.) Thesis, The University of Edinburgh.

Gauchat, J.F., Henchoz, S., Mazzei, G., Aubrey, J.P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L., Thompson, J., Kishi, K., Butterfield, J., Dahinden, C. and Bonnefoy, J.Y., 1993. Induction of human IgE synthesis in B-cells by mast cells and basophils. Nature, **365**: 340-343.

Ghiara, P., Boraschi, D., Villa, L., Scapigliati, G., Taddei, C. and Tagliabue, A., 1985. In vitro generated mast cells express natural cytotoxicity against tumour cells.
Immunology, **55**: 317-324.

Ghildyal, N., M^{c}Neil, H.P., Stechshulte, S., Austen, K.F., Silberstein, D., Gurish, M.F., Somerville, L.L. and Stevens, R.L., 1992. IL-10 induces transcription of the gene for mouse mast cell protease-1, a serine protease preferentially expressed in mucosal mast cells of *Trichinella spiralis* infected mice. Journal of Immunology, **149**: 2123-2129.

Gianetti, A. and Girolomoni, G., 1989. Skin reactivity to neuropeptides in atopic dermatitis.
British Journal of Dermatology, **121**: 681-688.

Giannetti, A., Fantini, F., Cimitan, A. and Pincelli, C., 1992. Vasoactive intestinal polypeptide and substance-P in the pathogenesis of atopic dermatitis.
Acta Dermato-Venereologica (Stockholm), S176, S16, 90-92.

Gibson, S.M. and Miller, H.R.P., 1986. Mast cell subsets in the rat distinguished immunohistochemically by their content of serine proteases. Immunology, **58**: 101-104.

Gibson, S., MacKellar, A., Newlands, G.F.J. and Miller, H.R.P., 1987. Phenotypic expression of mast cell granule proteases; distribution of mast cell proteinases I and II in the rat digestive system. Immunology, **62**: 621-628.

Gilead, L., Livni, N., Eliakim, R., Ligumsky, M., Fich, A., Okon, E., Rachmilewitz, D. and Razin, E., 1987. Human gastric mucosal mast cells are chondroitin sulphate E-containing cells. Immunology, **62**: 23-28.

Ginsburg, H., 1963. The *in vitro* differentiation and culture of normal mast cells from the mouse thymus. Annals of the New York Academy of Sciences, **103**: 20-39.

Ginsburg, H. and Lagunoff, D., 1967. The in vitro differentiation of mast cells. Cultures of cells from immunized mouse lymph nodes and thoracic duct lymph on fibroblast monolayers. The Journal of Cell Biology, **35**: 685-697.

Ginsburg, H., Ben-Shahar, D. and Ben-David, E., 1982. Mast cell growth on fibroblast monolayers: two cell entities. Immunology, **45**: 371-380.

Ginsburg, H., Amira, M., Padawar, J. and Davidson, S., 1989. Structural alterations in fibroblast monolayers caused by mast cell degranulation.
Journal of Leukocyte Biology, **45**: 491-497.

Glenner, G.G. and Cohen, L.A., 1960. Histochemical demonstration of a species-specific trypsin-like enzyme in mast cells. Nature, **185**: 846-847.

Goetzl, E.J., Chernov-Rogan, T., Furuichi, K., Goetzl, L.M., Lee, J.Y. and Renold, F., 1986. Neuromodulation of mast cell and basophil function. In: A.D.Befus, J.Bienenstock and J.A.Denburg (Editors), Mast Cell Differentiation and Heterogeneity. Raven Press Ltd., New York, pp. 223-229.

Goldstein, S.M., Wasserman, S.I. and Wintroub, B.U., 1989. Mast cell and eosinophil mediated damage in bullous pemphigoid. Immunology Series, **46**: 527-545.

Goldstein, S.M., Leong, J., Schwartz, L.B. and Cooke, D., 1992. Protease composition of exocytosed human skin mast cell protease-proteoglycan complexes. Tryptase resides in a complex distinct from chymase and carboxypeptidase.
Journal of Immunology, **148**: 2475-2482.

Gomori, G., 1953. Chloracyl esters as histochemical substrates.
Journal of Histochemistry and Cytochemistry, **1**: 469-470.

Gordon, J.R. and Galli, S.J., 1990a. Mast cells as a source of both pre-formed and immunologically-inducible TNF-α/cachectin. Nature, **346**: 274-276.

Gordon, J.R. and Galli, S.J., 1990b. Phorbol 12-myristate 13-acetate-induced development of functionally active mast cells in W/W^v but not Sl/Sl^d genetically mast cell-deficient mice. Blood, **75**: 1637-1645.

Gordon, J.R., Burd, P.R. and Galli, S.J., 1990. Mast cells as a source of multifunctional cytokines. Immunology Today, **11**: 458-464.

Gordon, J.R. and Galli, S.J., 1991. Release of both preformed and newly synthesized tumor necrosis factor α (TNF-α)/cachectin by mouse mast cells stimulated by the $Fc_{\varepsilon}RI$: a mechanism for the sustained action of mast cell-derived TNF-α during IgE-dependant biological responses. Journal of Experimental Medicine, **174**: 103-107.

Gordon, J.R. and Galli, S.J., 1994. Promotion of mouse fibroblast collagen gene-expression by mast cells stimulated via the FcεRI - role for mast cell-derived transforming growth factor-β and tumor necrosis factor-α. Journal of Experimental Medicine, **180**: 2027-2037.

Grabbe, J., Welker, P., Dippel, E. and Czarnetzki, B.M., 1994. Stem cell factor, a novel cutaneous growth factor for mast cells and melanocytes.
Archives of Dermatological Research, **287**: 78-84.

Grant, D.I. and Thoday, K.L., 1991. The skin. In: E.A.Chandler, D.J.Thompson, J.B.Sutton and C.J.Price (Editors), Canine Medicine and Therapeutics, 3rd Edition. Blackwell Scientific Publications, Oxford, pp. 365-416.

Green, R.M., Cordero, A. and Winklemann, R.M., 1977. Epidermal mast cells.
Archives of Dermatology, **113**: 166-169.

Grencis, R.K., Else, K.J., Huntley, J.F. and Nishikawa, S.I., 1993. The *in vivo* role of stem cell factor (*c-kit* ligand) on mastocytosis and host protective immunity to the intestinal nematode *Trichinella spiralis* in mice. Parasite Immunology, **15**: 55-59.

Gross, T.L. and Halliwell, R.E.W., 1985. Lesions of experimental flea bite hypersensitivity in the dog. Veterinary Pathology, **22**: 78-81.

Gross, T.L., Ihrke, P.J. and Walder, E.J., 1992. Veterinary Dermatopathology. A Macroscopic And Microscopic Evaluation Of Canine And Feline Skin Disease. Mosby Year Book, St. Louis, pp. 119-122.

Grossman, R.M., Kruegar, J., Yourish, D., Granellipiperno, A., Murphy, D.P., May, L.T., Kupper, T.S., Sehgal, P.B. and Gottlieb, A.B., 1989. Interleukin-6 is expressed in high-levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes.
Proceedings of the National Academy of Sciences of the USA, **86**: 6367-6371.

Groves, R.W., Allen, M.H., Ross, E.L., Barker, J.N.W.N., MacDonald, D.M., 1995. Tumour necrosis factor is pro-inflammatory in normal human skin and modulates cutaneous adhesion molecule expression. British Journal of Dermatology, **132**: 345-352.

Gruber, B.L., Marchese, M.J., Suzuki, K., Schwartz, L.B., Okada, Y., Nagase, H. and Ramamurthy, N.S., 1989. Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation.
Journal of Clinical Investigation, **84**: 1657-1662.

Gurish, M.F., Pear, W.S., Stevens, R.L., Scott, M.L., Sokol, K., Ghildyal, N., Webster, M.J., Hu, X., Austen, K.F., Baltimore, D. and friend, D.S., 1995. Tissue-regulated differentiation and maturation of a *v-abl*-immortalized mast cell-committed progenitor.
Immunity, **3**: 175-186.

Hägermark, Ö., Rajka, G. and Bergqvist, U., 1972. Experimental itch in human skin elicited by rat mast cell chymase. Acta Dermato-Venereologica (Stockholm), **52**: 125-128.

Hägermark, Ö., 1987. Skin as a response organ for neuropeptides.
Clinical and Experimental Dermatology, **12**: 136 (abstract).

Haig, D.M., 1993. Ovine bone-marrow stromal cell-dependent myelopoiesis.
Journal of Comparative Pathology, **109**: 259-270.

Haig, D.M., McKee, T.A., Jarrett, E.E.E., Woodbury, R. and Miller, H.R.P., 1982.
Generation of mucosal mast cells is stimulated *in vitro* by factors from T cells of
helminth-infected rats. Nature, **300**: 188-190.

Haig, D.M., M^{c}Menamin, C., Gunneberg, C., Woodbury, R.G. and Jarrett, E.E.E., 1983.
Stimulation of mucosal mast cell growth in normal and nude rat bone marrow cultures.
Proceedings of the National Academy of Sciences of the USA, **80**: 4499-4503.

Haig, D.M., M^{c}Menamin, C., Redmond, J., Brown, D., Young, I.G., Cohen, S.D. and
Hapel, A.J., 1988a. Rat IL-3 stimulates the growth of rat mucosal mast cells in culture.
Immunology, **65**: 205-211.

Haig, D.M., Blackie, W., Huntley, J., Mackellar, A. and Smith, W.D., 1988b. The generation
of ovine bone marrow-derived mast cells in culture. Immunology, **65**: 199-203.

Haig, D., Entrican, G., Yirrell, D., Deane, D., Miller, H.R.P., Norval, M. and
Reid, H.W., 1992. Differential appearance of interferon-γ and colony stimulating activity in
afferent versus efferent lymph following orf virus infection of sheep.
Veterinary Dermatology, **3**: 221-229.

Haig, D.M., Huntley, J.F., MacKellar, A., Newlands, G.F.J., Inglis, L., Sangha, R.,
Cohen, D., Hapel, A., Galli, S.J. and Miller, H.R.P., 1994. Effects of stem cell factor
(Kit-ligand) and interleukin-3 on the growth and serine proteinase expression of rat
bone-marrow-derived or serosal mast cells. Blood, **83**: 72-83.

Halliwell, R.E.W. and Schwartzman, R.M., 1971. Atopic disease in the dog.
The Veterinary Record, **89**: 209-214.

Halliwell, R.E.W., Schwartzman, R.M. and Rockey, J.H., 1972. Antigenic relationship
between human IgE and canine IgE. Clinical and Experimental Immunology, **10**: 399.

Halliwell, R.E.W. and Longino, S.J., 1985. IgE and IgG antibodies to flea antigen in differing
dog populations. Veterinary Immunology and Immunopathology, **8**: 215-223.

Hartley, B.S., 1960. Proteolytic enzymes. Annual Review of Biochemistry, **29**: 45-72.

Harvima, I.T., Schechter, N.M., Harvima, R.J. and Fräki, J.E., 1988. Human skin tryptase:
purification, partial characterization and comparison with human lung tryptase.
Biochemica et Biophysica Acta, **957**: 71-80.

Harvima, I.T., Naukkarinen, A., Harvima, R.J. and Horsmanheimo, M., 1989. Enzyme- and
immunohistochemical localization of mast cell tryptase in psoriatic skin.
Archives of Dermatological Research, **281**: 387-391.

Harvima, I.T., Naukkarinen, A., Paukkonen, K., Harvima, R.J., Aalto, M-L., Schwartz, L.B.
and Horsmanheimo, M., 1993. Mast cell tryptase and chymase in developing and mature
psoriatic lesions. Archives of Dermatological Research, **285**: 184-192.

Harvima, R.J. and Schwartz, L.B., 1993. Mast cell-derived mediators. In: J.C.Foreman (Editor), Immunopharmacology of Mast Cells and Basophils, Academic Press Limited, New York, pp. 115-138.

Harvima, I.T., Horsmanheimo, L., Naukkarinen, A. and Horsmanheimo, M., 1994. Mast cell proteinases and cytokines in skin inflammation.
Archives of Dermatological Research, **287**: 61-67.

Harvima, I.T., Harvima, R.J., Naukkarinen, A. and Horsmanheimo, M., 1995. Skin tryptase: features and expression in human dermatological disorders. In. G.H.Caughey (Editor), Mast Cell Proteases in Immunology and Biology, Marcel Dekker Inc., New York, pp. 25-46.

Haas, N., Hamann, K., Grabbe, J., Algermissen, B., Czarnetzki, B.M., 1995. Phenotypic characterization of skin lesions in urticaria pigmentosa and mastocytosis.
Archives of Dermatological Research, **287**: 242-248.

Hawk, J.L.M., Eady, R.A.J., Challoner, A.V.J., Kobza-Black, A., Keahey, T.M. and Greaves, M.W., 1980. Elevated blood histamine levels and mast cell degranulation in solar urticaria. British Journal of Clinical Pharmacology, **9**: 183-186.

He, S.H., McEuen, H.R., Brander, M. and Walls, A.F. (1995). Characterisation of guinea pig lung tryptase. Journal of Allergy and Clinical Immunology, **95**: 294.

Heavey, D.J., Kobza-Black, A., Barrow, S.E., Chappell, G.C., Greaves, M.W. and Dollery, C.T. (1986). Prostaglandin D_2 and histamine release in cold urticaria.
Journal of Allergy and Clinical Immunology, **78**: 458-461.

Hecker, J.F., 1974. Cannulation of lymphatics. In: Experimental Surgery On Small Ruminants. Butterworth & Co., Publishers Ltd., London, pp. 87-95.

Hellström, B. and Holmgren, H.J., 1950. Numerical distribution of mast cells in the human skin and heart. Acta Anatomica (Basel), **10**: 81.

Henfrey, J.I., Thoday, K.L. and Head, K.W., 1991. A comparison of three local anaesthetic techniques for skin biopsy in dogs. Veterinary Dermatology, **2**: 21-27.

Heyderman, E. and Neville, A.M., 1977. A shorter immunoperoxidase technique for the demonstration of carcinoembryonic antigen and other cell products.
Journal of Clinical Pathology, **30**: 138-140.

Holliday, M.R., Banks, E.M.S., Dearman, R.J., Kimber, I. and Coleman, J.W., 1994. Interactions of IFN-γ with IL-3 and IL-4 in the regulation of serotonin and arachidonate release from mouse peritoneal mast cells. Immunology, **82**: 70-74.

Homer, A.A., 1971. Macromolecular heparin from rat skin.
Journal of Biological Chemistry, **246**: 231-239.

Horsmanheimo, L., Harvima, I.T., Järvikallio, A., Harvima, R.J., Naukkarinen, A. and Horsmanheimo, M., 1994. Mast cells are one major source of interleukin-4 in atopic dermatitis. British Journal of Dermatology, **131**: 348-353.

Huang, E., Nocka, K., Beier, D.R., Chu, T-Y., Buck, J., Lahm, H-W., Wellner, D., Leder, P. and Besmer, P., 1990. The hematopoietic growth factor KL is encoded by the *Sl* locus and is the ligand of *c-kit* receptor, the gene product of the *W* locus. Cell, **63**: 225-233.

Huff, T.F., Lantz, C.S., Ryan, J.J. and Leftwich, J.A., 1995. Mast cell-committed progenitors. In: Y.Kitamura, S.Yamamoto, S.J.Galli and M.W.Greaves (Editors), Biological and Molecular Aspects of Mast Cell and Basophil Differentiation and Function. Raven Press, New York, pp.105-117.

Hültner, L., Moeller, J., Schmitt, E., Jäger, G., Reisbach, G., Ring, J. and Dörmer, P., 1989. Thiol-sensitive mast cell lines derived from mouse bone marrow respond to a mast cell growth-enhancing activity different from both IL-3 and IL-4.
Journal of Immunology, **142**: 3440-3446.

Hültner, L., Druez, C., Moeller, J., Uyttenhove, C., Schmitt, E., Rüde, E., Dörmer, P. and Van Snick, J., 1990. Mast cell-growth enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (Interleukin 9)*.
European Journal of Immunology, **20**: 1413-1416.

Humphrey, D.M., M^{c}Manus, L.M., Hanahan, D.J. and Pinckard, R.N., 1984. Morphologic basis of increased vascular permeability induced by acetyl glyceryl ether phosphorylcholine.
Laboratory Investigation, **50**: 16-25.

Hunt, T.C., Campbell, A.M., Robinson, C. and Holgate, S.T., 1991. Structural and secretory characteristics of bovine lung and skin mast cells: evidence for the existence of heterogeneity.
Clinical and Experimental Allergy, **21**: 173-182.

Huntley, J.F., 1991. Mast cells and intestinal nematodiasis. PhD Thesis, The University of Edinburgh.

Huntley, J.F., 1992. Mast cells and basophils: a review of their heterogeneity and function.
Journal of Comparative Pathology, **107**: 349-372.

Huntley, J.F., 1995. *Personal communication.*

Huntley, J.F., Wallace, G.R. and Miller, H.R.P., 1982. Quantitative recovery of isolated mucosal mast cells and globule leucocytes from parasitised sheep.
Research in Veterinary Science, **33**: 58-63.

Huntley, J.F., Newlands, G. and Miller, H.R.P., 1984. The isolation and characterization of globule leucocytes: their derivation from mucosal mast cells in parasitized sheep.
Parasite Immunology, **6**: 371-390.

Huntley, J.F., Newlands, G.F.J., Gibson, S., Ferguson, A. and Miller, H.R.P., 1985. Histochemical demonstration of chymotrypsin like serine esterases in mucosal mast cells of four species including man. Journal of Clinical Pathology, **38**: 375-384.

Huntley, J.F., Gibson, S., Knox, D. and Miller, H.R.P., 1986. The isolation and purification of a proteinase with chymotrypsin-like properties from ovine mucosal mast cells.
International Journal of Biochemistry, **18**: 673-682.

Huntley, J.F., Gibson, S., Brown, D., Smith, W.D., Jackson, F. and Miller, H.R.P., 1987. Systemic release of a mast cell proteinase following nematode infections in sheep.
Parasite Immunology, **9**: 603-614.

Huntley, J.F., MacKellar, A., Newlands, G.F., Irvine, J. and Miller, H.R.P., 1990. Mapping of the rat mast cell granule proteinases RMCP I and II by enzyme-linked immunosorbent assay and paired immunofluorescence.
Acta Pathologica, Microbiologica, et Immunologica Scandinavica, **98**: 933-944.

Huntley, J.F., Haig, D.M., Irvine, J., Inglis, L., MacDonald, A., Rance, A. and Moqbel,R., 1992. Characterisation of ovine mast cells derived from in vitro culture of haemopoietic tissue. Veterinary Immunology and Immunopathology, **32**: 47-64.

Huntley, J.F., MacKellar, A. and Miller, H.R.P., 1993. Altered expression of mast cell proteases in the rat. Quantitative and immunohistochemical analysis of the distribution of rat mast cell proteases I and II during helminth infection.
Acta Pathologica, Microbiologica, et Immunologica Scandinavica, **101**: 953-962.

Iakovleva, A.I., 1954. The function of adipose cells in the connective tissue.
Morfologia Patomorfologia, **40**: 70.

Ihle, J.N., Keller, J., Oroszlan, S., Henderson, L.E., Copeland, T.D., Fitch, F., Prystowsky, M.B., Goldwasser, E., Schrader, J.W., Palaszynski, E., Dy, M. and Lebel, B., 1983. Biological properties of homogenous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity.
Journal of Immunology, **131**: 282-287.

Irani, A.A., Schechter, N.M., Craig, S.S., DeBlois, G. and Schwartz, L.B., 1986. Two types of human mast cells that have distinct neutral protease compositions.
Proceedings of the National Academy of Sciences of the USA, **83**: 4464-4468.

Irani, A.A. and Schwartz, L.B., 1990. Neutral proteases as indicators of human mast cell heterogeneity. Monographs in Allergy, **27**: 146-162.

Irani, A.A., Garriga, M.M., Metcalfe, D.D. and Schwartz, L.B., 1990. Mast cells in cutaneous mastocytosis: accumulation of the MC_{TC} type.
Clinical and Experimental Allergy, **20**: 53-58.

Irani, A.A., Craig, S.S., Nilsson, G., Ishizaka, T., Schwartz, L.B., 1992. Characterization of human mast cells developed *in vitro* from fetal liver cells cocultured with murine 3T3 fibroblasts. Immunology, **77**: 136-143.

Ishizaka, T., Okudaira, H., Mauser, L.E. and Ishizaka, K., 1976. Development of rat mast cells *in vitro*. I. Differentiation of mast cells from thymus cells.
Journal of Immunology, **116**: 747-754.

Ishizaka, T., Adachi, T., Chang, T.-H. and Ishizaka, K., 1977. Development of rat mast cells *in vitro*. II. Biologic function of cultured mast cells. Journal of Immunology, **118**: 211-217.

Ishizaka, T., Furitsu, T. and Inagaki, N., 1991. In vitro development and functions of human mast cells. International Archives of Allergy and Applied Immunology, **94**: 116-121.

Issekutz, A.C., 1981. Vascular responses during acute neutrophilic inflammation. Their relationship to *in vivo* neutrophil emigration. Laboratory Investigation, **45**: 435-441.

Iwamoto, I., Tomoe, S., Tomioka, H. and Yoshida, S., 1992. Substance P-induced granulocyte infiltration in mouse skin: the mast cell-dependent granulocyte infiltration by the N-terminal peptide is enhanced by the activation of vascular endothelial cells by the C-terminal peptide. Clinical and Experimental Immunology, **87**: 203-207.

Jacques, P., Lavoie, A., Bedard, P.M., Brunet, C. and Hebert, J., 1992. Chronic idiopathic urticaria: profiles of skin mast cell histamine release during active disease and remission.
Journal of Allergy and Clinical Immunology, **89**: 1139-1143.

Jenkinson, D.M., Thompson, G.E., Kenny, J.D.R., and Pearson, J.M., 1970. Histochemical studies on mast cells in cattle skin. Histochemical Journal, **2**: 419-424.

Johnson, A.R. and Moran, N.C., 1969. Selective release of histamine from rat mast cells by compound 48/80 and antigen. American Journal of Physiology, **216**: 453-459.

Johnson, D.A. and Cawston, T.E., 1985. Human lung mast cell tryptase fails to activate procollagenase or degrade proteoglycan.
Biochemical and Biophysical Research Communications, **132**: 453-459.

Kaminer, M.S., Lavker, R.M., Walsh, L.J., Whitaker, D., Zweiman, B. and Murphy, G.F., 1991. Extracellular localization of human connective tissue mast cell granule contents.
Journal of Investigative Dermatology, **96**: 857-863.

Katz, H.R., Stevens, R.L. and Austen, K.F., 1985. Heterogeneity of mammalian mast cells differentiated in vivo and in vitro. Journal of Allergy and Clinical Immunology, **76**: 250-259.

Kaufman, A. and Rosenstreich, D.L., 1990. Mast cell heterogeneity in chronic idiopathic urticaria. Annals of Allergy, **65**: 367-373.

Kay, A.B., Ying, S., Varney, V., Gaga, M., Durham, S.R., Moqbel, R., Wardlaw, A.J. and Hamid, Q., 1991. Messenger-RNA expression of the cytokine gene-cluster, interleukin 3 (IL-3), IL-4, IL-5 and granulocyte macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects.
Journal of Experimental Medicine, **173**: 775-778.

Kieffer, M. and Kristensen, S., 1979. Flea hypersensitivity in dogs and cats.
International Journal of Dermatology, **18**: 707-712.

Kirkwood, A.C., 1980. Effect of *P.ovis* on the weight of sheep.
The Veterinary Record, **107**: 469-470.

Kirshenbaum, A.S., Goff, J.P., Dreskin, S.C., Irani, A.M., Schwartz, L.B. and Metcalfe, D.D., 1989. IL-3-dependent growth of basophil-like cells and mastlike cells from human bone marrow. Journal of Immunology, **142**: 2424-2429.

Kirshenbaum, A.S., Goff, J.P., Kessler, S.W., Mican, J.M., Zsebo, K.M. and Metcalfe, D.D., 1992. Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells. Journal of Immunology, **148**: 772-777.

Kitamura, Y., Shimada, M., Hatanaka, M. and Miyano, Y., 1977. Development of mast cells from grafted bone marrow cells in irradiated mice. Nature, **268**: 442-443.

Kitamura, Y., Go, S. and Hatanaka, K., 1978. Decrease of mast cells in W/W^v mice and their increase by bone marrow transplantation. Blood, **52**: 447-452.

Kitamura, Y. and Go, S., 1979. Decreased production of mast cells in Sl/Sl^d anemic mice. Blood, **53**: 492-497.

Kitamura, Y., Matsuda, H. and Hatanaka, K. 1979. Clonal nature of mast cell clusters in W/W^v mice after bone marrow transplantation. Nature, **281**: 154-155.

Kitamura, Y., Hatanaka, K., Murakami, M. and Shibata, H., 1979. Presence of mast cell precursors in peripheral blood of mice demonstrated by parabiosis. Blood, **53**: 1085-1088.

Kitamura, Y., Kanakura, Y., Sonoda, S., Asai, H. and Nakano, T., 1987. Mutual phenotypic changes between connective tissue type and mucosal mast cells.
International Archives of Allergy and Applied Immunology, **82**: 244-248.

Kjellen, L. and Lindahl, U., 1991. Proteoglycans: structures and interactions.
Annual Reviews of Biochemistry, **60**: 443-475.

Klein, L.M., Lavker, R.M. and Murphy, G.F., 1989. An in vitro model for mast cell degranulation in intact human skin. Journal of Investigative Dermatology, **92**: 460.

Klein, L.M., Lavker, R.M., Matis, W.L. and Murphy, G.F., 1989. Degranulation of human mast cells induces an endothelial antigen central to leukocyte adhesion.
Proceedings of the National Academy of Sciences of the USA, **86**: 8972-8976.

Knox, D.P., Gibson, S. and Huntley, J.F., 1986. The catalytic properties of a proteinase isolated from sheep abomasal mucosal mast cells.
International Journal of Biochemistry, **18**: 961-964.

Knox, D.P. and Huntley, J.F., 1987. Classification of sheep abomasal mucosal mast cell proteinase as a serine endopeptidase (EC 3.4.21).
International Journal of Biochemistry, **20**: 193-195.

Kobza-Black, A., Keahey, T.M., Bentley-Philips, C.M., Eady, R.A., Sibbald, G. and Greaves, M.W., 1979. Release of histamine in cold urticaria. In: J.Pepys and A.M.Edwards (Editors), The Mast Cell - It's Role In Health and Disease. Pitman Publishing, Marshfield, M.A., pp. 607.

Kowalski, M.L. and Kaliner, M.A., 1988. Neurogenic inflammation, vascular permeability and mast cells. Journal of Immunology, **140**: 3905-3911.

Kozlowski, G.P. and Calhoun, M.L., 1969. Microscopic anatomy of the integument of sheep. American Journal of Veterinary Research, **30**: 1267-1279.

Krogsgaard Thomsen, M., 1991. The role of neutrophil-activating mediators in canine health and disease (with special reference to the role of leukotrienes in inflammatory dermatoses). Journal of Veterinary Pharmacology and Therapeutics, **14**: 113-133.

Kupper, T.S., Schechter, N., Lazarus, G. and Mizutani, H., 1990. Rapid and specific conversion of inactive precursor IL-1 beta to mature active IL-1 by human mast cell chymase: a role for mast cell proteases in the initiation of inflammatory responses.
Journal of Investigative Dermatology, **94**: 545.

Kupper, T.S. and Groves, R.W., 1995. The interleukin-1 axis and cutaneous inflammation. Journal of Investigative Dermatology, **105**: 62S-66S.

Kyan Aung, U., Haskard, D.O., Poston, R.N., Thornhill, M.H. and Lee, T.H., 1991. Endothelial leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 mediate the adhesion of eosinophils to endothelial cells *in vitro* and are expressed by endothelium in allergic cutaneous inflammation *in vivo*. Journal of Immunology, **146**: 521-528.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680-685.

Lagunoff, D. and Benditt, E.P., 1961. Histochemical examinations of chymotrypsin-like esterases. Nature, **192**: 1198-1199.

Lagunoff, D., Benditt, E.P. and Watts, R.M., 1962. Histochemical study of esterases homospecific with trypsin. Journal of Histochemistry and Cytochemistry, **10**: 672-673.

Lagunoff, D. and Benditt, E.P., 1963. Proteolytic enzymes of mast cells. Annals of the New York Academy of Sciences, **103**: 185-198.

Lagunoff, D. and Pritzl, P., 1976. Characterization of rat mast cell granule proteins. Archives of Biochemistry and Biophysics, **173**: 554-563.

Lagunoff, D., Rickard, A. and Marquardt, C., 1991. Rat mast cell tryptase. Archives of Biochemistry and Biophysics, **291**: 52-58.

Lawrence, I.D., Warner, J.A., Cohan, V.L., Hubbard, W.C., Kagey-Sobotka, A. and Lichtenstein, L.M., 1987. Purification and characterization of human skin mast cells. Evidence for human mast cell heterogeneity. The Journal of Immunology, **139**: 3062-3069.

Le, J. and Vilcek, J., 1987. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Laboratory Investigation, **56**: 234-248.

Le Trong, H., Neurath, H. and Woodbury, R.G., 1987. Substrate specificity of the chymotrypsin-like protease in secretory granules isolated from rat mast cells. Proceedings of the National Academy of Sciences of the USA, **84**: 364-367.

Le Trong, H., Newlands, G.F.J., Miller, H.R.P., Charbonneau, H., Neurath, H. and Woodbury, R.G., 1989. Amino acid sequence of a mouse mucosal mast cell protease. Biochemistry, **28**: 391-395.

Leung, K.B.P., Flint, K.C., Hudspith, B.N., Brostoff, J., Johnson, N.M., Seager, K., Hammond, M.D. and Pearce, F.L., 1987. Some further properties of human pulmonary mast cells recovered by bronchoalveolar lavage and enzyme dispersion of lung tissue. Agents and Actions, **20**: 213-215.

Levi-Schaffer, F., Austen, K.F., Caulfield, J.P., Hein, A., Bloes, W.F. and Stevens, R.L., 1985. Fibroblasts maintain the phenotype and viability of the rat heparin-containing mast cell in vitro. Journal of Immunology, **135**: 3454-3462.

Levi-Schaffer, F., Austen, K.F., Gravallese, P.M. and Stevens, R.L., 1986. Coculture of interleukin 3-dependent mouse mast cells with fibroblasts results in a phenotypic change of the mast cells. Proceedings of the National Academy of Sciences of the USA, **83**: 6485-6488.

Levi-Schaffer, F. and Riesel, N., 1989. *In vitro* regeneration of activated rat peritoneal mast cells cocultured with 3T3 fibroblasts. Cellular Immunology, **119:** 30-40.

Levi-Schaffer, F. and Rubinchik, E., 1995. Activated mast cells are fibrogenic for 3T3 fibroblasts. Journal of Investigative Dermatology, **104**: 999-1003.

Lewis, R.A. and Austen, K.F., 1981. Mediation of local homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. Nature, **293**: 103-108.

Lewis, R.A., Soter, N.A., Diamond, P.T., Austen, K.F., Oates, J.A. and Roberts, L.J., 1982. Prostaglandin D_2 generation after activation of rat and human mast cells with anti-IgE. Journal of Immunology, **129**: 1627-1631.

Lichtenstein, L.M., Foreman, J.C., Conroy, M.C., Marone, G. and Newball, H.H., 1979. Differences between histamine release from rat mast cells and human basophils and mast cells. In: J. Pepys and A.M. Edwards, (Editors), The Mast Cell Its Role in Health and Disease. Pitman Medical, Tunbridge Wells, pp. 83-96.

Lloyd, A.R. and Oppenheim, J.J., 1992. Poly's lament : the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. Immunology Today, **13**: 169-172.

Longley, B.J., Morganroth, G.S., Tyrrell, B.A., Ding, T.G., Anderson, D.M., Williams, D.E. and Halaban, R., 1992. Altered metabolism of mast cell growth factor (*c-kit* ligand) in cutaneous mastocytosis. New England Journal of Medicine, **328**: 1302-1307.

Lopez, A.F., Elliott, M.J., Woodcock, J. and Vadas, M.A., 1992. GM-CSF, IL-3 and IL-5: cross competition on human haemopoietic cells. Immunology Today, **13**: 495-500.

Lowman, M.A., Benyon, R.C. and Church, M.K., 1988. Characterization of neuropeptide-induced histamine release from dispersed skin mast cells. British Journal of Pharmacology, **95**: 121-130.

Lowman, M.A., Rees, P.H., Benyon, R.C. and Church, M.K., 1988. Human mast cell heterogeneity: histamine from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependant and nonimmunologic stimuli. Journal of Allergy and Clinical Immunology, **81**: 590-597.

Lucio, J., D'Brot, J., Guo, C.B., Abraham, W.M., Lichtenstein, L.M., Kageysobotka, A. and Ahmed, T., 1992. Immunologic mast cell-mediated responses and histamine release are attenuated by heparin. Journal of Applied Physiology, **73**: 1093-1101.

Luger, T.A. and Schwarz, T., 1990. Epidermal cell-derived cytokines. In: J.D.Bos (Editor), Skin Immune System. CRC Press Inc., Florida, pp. 257-291.

MacDonald, A.J., Haig, D.M., Bazin, H., M^{c}Guigan, A.C., Moqbel, R. and Miller, H.R.P., 1989. IgE-mediated release of rat mast cell protease II, β-hexosaminidase and leukotriene C_4 from culture bone marrow-derived rat mast cells. Immunology, **67**: 414-418.

MacDonald, A.J., (1994). Studies on mediator release by cultured rat bone marrow-derived mast cells: potential relevance to helminth-induced intestinal inflammation. PhD Thesis, University of London.

MacKay, C.R. and Imhof, B.A., 1993. Cell adhesion in the immune system. Immunology Today, **14**: 99-102.

Marshall, J.S., Ford, G.P. and Bell, E.B., 1987. Formalin sensitivity and differential staining of mast cells in human dermis. British Journal of Dermatology, **117**: 29-36.

Marshall, J.S. and Bienenstock, J., 1994. The role of mast cells in inflammatory reactions of the airways, skin and intestine. Current Opinion in Immunology, **6**: 853-859.

Martin, W.B. and Aitken, I.D. [Editors] (1991). Diseases of Sheep, 2nd Edition, pp. 261-298.

Massey, W.A., Guo, C.B., Dvorak, A.M., Hubbard, W.C., Bhagavan, B.S., Cohan, V.L., Warner, J.A., Kagey-Sobotka, A. and Lichtenstein, L.M., 1991. Human uterine mast cells. Isolation, purification, characterisation, ultrastructure and pharmacology. Journal of Immunology, **147**: 1621-1627.

Matsuda, H., Fukui, K., Kiso, Y. and Kitamura, Y., 1985. Inability of genetically mast cell-deficient *W/W^v* mice to acquire resistance against larval *Haemaphysalis longicornis* ticks. Journal of Parasitology, **71**: 443-448.

Matsuda, H., Nakano, T., Kiso, Y. and Kitamura, Y., 1987. Normalization of anti-tick response of mast cell-deficient *W/W^v* mice by intracutaneous injection of cultured mast cells. Journal of Parasitology, **73**: 155-160.

Matsuda, H., Kawakita, K., Kiso, Y., Nakano, T. and Kitamura, Y., 1989. Substance P induces granulocyte infiltration through degranulation of mast cells. Journal of Immunology, **142**: 927-931.

Matsuda, H., Watanabe, N., Kiso, Y., Hirota, S., Ushio, H., Kannan, Y., Azuma, M., Koyama, H. and Kitamura, Y., 1990. Necessity of IgE antibodies and mast cells for manifestation of resistance against larval *Haemaphysalis longicornis* ticks in mice. Journal of Immunology, **144**: 259-262.

Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H. and Kitamura, Y., 1991. Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. Journal of Experimental Medicine, **174**: 7-14.

Matsumoto, R., Sali, A., Ghildyal, N., Karplus, M. and Stevens, R.L., 1995. Packaging of proteases and proteoglycans in the granules of mast cells and other hematopoietic cells - a cluster of histidines on mouse mast cell protease-7 regulates its binding to heparin serglycin proteoglycans. Journal of Biological Chemistry, **270**: 19524-19531.

Mayrhofer, G., 1979. The nature of the thymus dependency of mucosal mast cells. II. The effect of thymectomy and of depleting recirculating lymphocytes on the response to *Nippostrongylus brasiliensis*. Cellular Immunology, **47**: 312-322.

Mayrhofer, G. and Fisher, R., 1979. Mast cells in severely T-cell depleted rats and the response to infestation with *Nippostrongylus brasiliensis*. Immunology, **37**: 145-155.

M^{c}Euen, A.R., Sharma, B. and Walls, A.F., 1995. Regulation of the activity of human chymase during storage and release from mast cells: the contributions of inorganic cations, pH, heparin and histamine. Biochemica et Biophysica Acta, **1267**: 115-121.

M^{c}Ewan Jenkinson, D., Hutchison, G. and Reid, H.W., 1992. The B and T cell responses to Orf virus infection of ovine skin. Veterinary Dermatology, **3**: 57-64.

M^{c}Innes, C., 1993. Current research on ovine cytokines. British Veterinary Journal, **149**: 371-386.

M^{c}Innes, C., 1995. *Personal communication.*

M^{c}Innes, C., Haig, D.M. and Logan, M., 1993. The cloning and expression of the gene for ovine interleukin-3 (multi-CSF) and a comparison of the in vitro haemopoietic activity of ovine IL-3 with ovine GM-CSF and human GM-CSF. Experimental Haematology, **21**: 1528-1534.

M^{c}Kay, I.A. and Leigh, I.M., 1991. Epidermal cytokines and their roles in cutaneous wound healing. British journal of Dermatology, **124**: 513-518.

M°Menamin, C., Haig, D.M., Gibson, S., Newlands, G.F. and Miller, H.R., 1987. Phenotypic analysis of mast cell granule proteinases in normal rat bone marrow cultures. Immunology, **60**: 147-149.

M°Neil, H.P., Austen, K.F., Somerville, L.L., Gurish, H.F. and Stevens, R.L., 1991. Molecular-cloning of the mouse mast cell protease-5 gene - a novel secretory granule protease expressed early in the differentiation of serosal mast cells. Journal of Biological Chemistry, **266**: 20316-20322.

M°Neil, H.P., Reynolds, D.S., Schiller, V., Ghildyal, N., Gurley, D.S., Austen, K.F. and Stevens, R.L., 1992. Isolation, characterisation, and transcription of the gene encoding mouse mast cell protease 7. Proceedings of the National Academy of Sciences of the USA, **89**: 11174-11178.

Meade, R., Neddermann, K.M., Greenfield, R.S., Braslawsky, G., Bursuker, I., 1993. Granulocyte-macrophage colony-stimulating factor plays a role in the functional activity of mast cells. Journal of Leukocyte Biology, **54**: 523-527.

Mekori, Y.A. and Galli, S.J., 1990. [^{125}I] Fibrin deposition occurs at both early and late intervals of IgE-dependent or contact sensitivity reactions elicited in mouse skin. Mast cell-dependent augmentation of fibrin deposition at early intervals in combined IgE-dependent and contact sensitivity reactions. Journal of Immunology, **145**: 3719-3727.

Metcalf, D., 1993. Haematopoietic regulators: redundancy or subtlety? Blood, **82**: 3515-3523.

Metcalfe, D.D., Soter, N.A., Wasserman, S.I. and Austen, K.F., 1980. Identification of sulfated mucopolysaccharides including heparin in the lesional skin of a patient with mastocytosis. Journal of Investigative Dermatology, **74**: 210-215.

Migliaccio, G., Migliaccio, A.R., Valinsky, J., Langley, K., Zsebo, K., Visser, J.W.M. and Adamson, J.W., 1991. Stem cell factor induced proliferation and differentiation of highly enriched murine haematopoietic cells. Proceedings of the National Academy of Sciences of the USA, **88**: 7420-7424.

Miller, H.R.P., Jackson, F., Newlands, G. and Appleyard, W.T., 1983. Immune exclusion, a mechanism of protection against the ovine nematode *Haemonchus contortus*. Research in Veterinary Science, **35**: 357-363.

Miller, H.R.P., Huntley, J.F., Newlands, G.F., MacKellar, A., Lammas, A.D. and Wakelin, D., 1988. Granule proteinases define mast cell heterogeneity in the serosa and the gastrointestinal mucosa of the mouse. Immunology, **65**: 559-566.

Miller, H.R.P., Huntley, J.F., Newlands, G.F.J., MacKellar, A., Irvine, J., Haig, D.M., MacDonald, A., Lammas, A.D., Wakelin, D. and Woodbury, R.G., 1989. Mast cell granule proteases in mouse and rat: a guide to mast cell heterogeneity and activation in the gastrointestinal tract. In: S.J.Galli and K.F.Austen (Editors), Mast Cell and Basophil Differentiation and Function in Health and Disease. Raven Press Ltd., New York, pp. 81-91.

Miller, H.R.P., 1993a. Mast cells in the gastrointestinal tract. In: J.C.Foreman (Editor), Immunopharmacology of Mast Cells and Basophils, Academic Press Limited, New York, pp. 197-215.

Miller, H.R.P., 1993b. Immunopathology of gastrointestinal nematode infestation and expulsion. Current Opinion in Gastroenterology, **9**: 986-993.

Miller, H.R.P., Huntley, J.F. and Newlands, G.F.J., 1995. Mast cell chymases in helminthosis and hypersensitivity. In: G.H.Caughey (Editor), Mast Cell Proteases in Immunology and Biology, Marcel Dekker Inc., New York, pp. 203-235.

Miller, J.S., Westin, E.H. and Schwartz, L.B., 1989. Cloning and characterization of complementary DNA for human tryptase. Journal of Clinical Investigation, **84**: 1188-1195.

Miller, J.S., Moxley, G. and Schwartz, L.B., 1990. Cloning and characterization of a second complementary DNA for human tryptase. Journal of Clinical Investigation, **86**: 864-870.

Mitsui, H., Furitsu, T., Dvorak, A.M., Irani, A.M.A., Schwartz, L.B., Inagaki, N., Takei, M., Ishizaka, K., Zsebo, K.M., Gillis, S. and Ishizaka, T., 1993. Development of human mast cells from umbilical cord blood cells by recombinant human and murine c-kit ligand. Proceedings of the National Academy of Sciences of the USA, **90**: 735-740.

Mizutani, H., Black, R. and Kupper, T.S., 1991. Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Journal of Clinical Investigation, **87**: 1066-1071.

Mizutani, H., Schechter, N., Lazarus, G., Black, R.A. and Kupper, T.S., 1991. Rapid and specific conversion of precursor interleukin 1β (IL-1β) to an active IL-1 species by human mast cell chymase. Journal of Experimental Medicine, **174**: 821-825.

Moeller, J., Hültner, L., Schmitt, E. and Dörmer, P., 1989. Partial purification of a mast cell growth-enhancing activity and its separation from IL-3 and IL-4. Journal of Immunology, **142**: 3447-3451.

Molinari, J.F., Moore, W.R., Clark, J., Tanaka, R., Butterfield, J.H. and Abraham, W.M. Role of tryptase in immediate cutaneous responses in allergic sheep. Journal of Applied Physiology, *in press*.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology, **136**: 2348-2357.

Mosmann, T.R., Schumacher, J.H., Street, N.F., Budd, R.C., O'Garra, A., Fong, T.A.T., Bond, M.W., Moore, K.W., Sher, A. and Fiorentino, D.F., 1991. Diversity of cytokine synthesis and function of mouse CD4+ T cells. Immunological Reviews, **123**: 209-229.

Mousli, M., Hugli, T.E., Landry, Y. and Bronner, C., 1994. Peptidergic pathway in human skin and rat peritoneal mast cell activation. Immunopharmacology, **27**: 1-11.

Mulder, K. and Colditz, I.G., 1993. Migratory responses of ovine neutrophils to inflammatory mediators in vitro and in vivo. Journal of Leukocyte Biology, **53**: 273-277.

Murakami, M., Austen, K.F. and Arm, J.P., 1995. Cytokine regulation of arachidonic acid metabolism in mast cells. In: Y.Kitamura, S.Yamamoto, S.J.Galli and M.W.Greaves (Editors), Biological and Molecular Aspects of Mast Cell and Basophil Differentiation and Function. Raven Press, New York, pp.25-37.

Myles, A.D., Halliwell, R.E.W., Ballauf, B. and Miller, H.R.P., 1995. Mast cell tryptase levels in normal canine tissues. Veterinary Immunology and Immunopathology, **46**: 223-235.

Nabel, G., Galli, S.J., Dvorak, A.M., Dvorak, A.F. and Cantor, H., 1981. Inducer T lymphocytes synthesize a factor that stimulates proliferation of cloned mast cells. Nature, **291**: 332-334.

Nadel, J.A., 1991. Biology of mast cell tryptase and chymase. Annals of the New York Academy of Sciences, **629**: 319-331.

Nagao, K., Yokoro, K. and Aaronson, S.A., 1981. Continuous lines of basophil/mast cells derived from normal mouse bone marrow. Science, **212**: 333-335.

Nakagawa, N., Sano, H. and Iwamoto, I., 1995. Substance P induces the expression of intercellular adhesion molecule-1 on vascular endothelial cells and induces neutrophil transendothelial migration. Peptides, **16**: 721-725.

Nakamura, K., Tanaka, T., Morita, E., Kameyoshi, Y., and Yamamoto, S., 1994. Enhancement of fibroblast-dependent mast cell growth in mice by a conditioned medium of keratinocyte-derived squamous cell carcinoma cells. Archives of Dermatological Research, **287**: 91-96.

Nakane, P.K. and Kawaoi, A.J., 1974. Peroxidase-labelled antibody. A new method of conjugation. Journal of Histochemistry and Cytochemistry, **22**: 1084-1091.

Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y. and Galli, S.J., 1985. Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/W^v mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells. Journal of Experimental Medicine, **162**: 1025-1043.

Nakano, T., Kanakura, Y., Nakahata, T., Matsuda, H. and Kitamura, Y., 1987. Genetically mast cell deficient W/W^v mice as a tool for studies of differentiation and function of mast cells. Federation Proceedings, **46**: 1920-1923.

Naukkarinen, A., Harvima, I.T., Aalto, M-L, and Horsmanheimo, M., 1994. Mast cell tryptase and chymase are potential regulators of neurogenic inflammation in psoriatic skin. International Journal of Dermatology, **33**: 361-366.

Nawa, Y. and Miller, H.R.P., 1979. Adoptive transfer of the intestinal mast cell response in rats infected with *Nippostrongylus brasiliensis*. Cellular Immunology, **42**: 225-239.

Nawa, Y., Horii, Y., Okada, M. and Arizono, N., 1994. Histochemical and cytological characterizations of mucosal and connective-tissue mast-cells of mongolian gerbils (*Meriones-unguiculatus*). International Archives of Allergy and Immunology, **104**: 249-254.

Newlands, G.F.J., Huntley, J.F. and Miller, H.R.P., 1984. Concomitant detection of mucosal mast cells and eosinophils in the intestines of normal and *Nippostrongylus*-immune rats. Histochemistry, **81**: 585-589.

Newlands, G.F.J., Gibson, S., Knox, D.P., Grencis, R., Wakelin, D. and Miller, H.R.P., 1987. Characterisation and mast cell origin of a chymotrypsin-like proteinase isolated from intestines of mice infected with *Trichinella spiralis*. Immunology, **62**: 629-634.

Newlands, G.F., Lammas, D.A., Huntley, J.F., MacKellar, A., Wakelin, D. and Miller, H.R., 1991. Heterogeneity of murine bone marrow-derived mast cells: analysis of their proteinase content. Immunology, **72**: 434-439.

Newlands, G.F.J., Knox, D.P., Pirie-Shepherd, S.R. and Miller, H.R.P., 1993. Biochemical and immunological characterization of multiple glycoforms of mouse mast cell proteinase-1 - comparison with an isolated murine serosal mast cell protease (MMCP-4). Biochemical Journal, **294**: 127-135.

Newlands, G.F.J., Miller, H.R.P., MacKellar, A. and Galli, S.J., 1995. Stem cell factor contributes to intestinal mucosal mast cell hyperplasia in rats infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis*, but anti-stem cell factor treatment decreases parasite egg-production during *N.brasiliensis* infection. Blood, **86**: 1968-1976.

Nickoloff, B.J., 1990. Adhesion molecules and inflammatory cell migration pathways in the skin. In: J.D.Bos (Editor), Skin Immune System. CRC Press Inc., Florida, pp. 49-71.

Nimmo Wilkie, J.S., Yager, J.A., Eyre, P. and Parker, W.M., 1990. Morphometric analyses of the skin of dogs with atopic dermatitis and correlations with cutaneous and plasma histamine and total serum IgE. Veterinary Pathology, **27**: 179-186.

Nishioka, K., Kobayashi, Y., Katayama, I. and Takajiri, C., 1987. Mast cell numbers in diffuse scleroderma. Archives of Dermatology, **123**: 205-208.

Nitsos, I. and Rees, S., 1993. Development of immunoreactivity for calcitonin gene-related peptide, substance P and glutamate in primary sensory neurones, and for serotonin in the spinal cord of fetal sheep. Neuroscience, **54**: 239-252.

Nocka, K., Buck, J., Levi, E. and Besmer, P., 1990. Candidate ligand for the *c-kit* transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. The EMBO Journal, **9**: 3287-3294.

Ogawa, M., 1993. Differentiation and proliferation of hematopoietic stem cells. Blood, **81**: 2844-2853.

Ohkubo, T., Shibata, M., Inoue, M., Kaya, H. and Takahashi, H., 1994. Autoregulation of histamine release via the histamine H_3 receptor on mast cells in the rat skin. Archives Internationales de Pharmacodynamie et de Therapie, **328**: 301-314.

Olafsson, J.H., Roupe, G. and Enerbäck, L., 1986. Dermal mast cells in mastocytosis: fixation, distribution and quantitation. Acta Dermatologica Venereologica (Stockholm), **66**: 16-22.

Oliver, F., Amon, E.U., Breathnach, A., Francis, D.M., Sarathchandra, P., Black, A.K. and Greaves, A.M., 1991. Contact urticaria due to the common stinging nettle (Urtica dioica) - histological, ultrastructural and pharmacological studies. Clinical and Experimental Dermatology, **16**: 1-7.

Orange, R.P. and Austen, K.F., 1972. Immunologic and pharmacologic receptor control of the release of chemical mediators from human lung. In: K.Ishizaka and D.H.Dayton (Editors), The Biological Role of the Immunoglobulin E System. National Institutes of Health, U.S. Department of Health, Education and Welfare, Bethesda, Md.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi Rosso, G. and Lobb, R., 1989. Direct expression cloning of vascular cell-adhesion molecule-1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell, **59**: 1203-1211.

Pasyk, K.K. and Cherry, G.W., 1990. Endothelial cells. In: J.D.Bos (Editor), Skin Immune System. CRC Press Inc., Florida, pp. 159-170.

Paul, W.E. and Seder, R.A., 1994. Lymphocyte responses and cytokines. Cell, **76**: 241-251.

Pearce, F.L., 1986. Functional differences between mast cells from various locations. In: A.D.Befus, J.Bienenstock and J.A.Denburg (Editors), Mast Cell Differentiation and Heterogeneity. Raven Press Ltd., New York, pp 215-222.

Pejler, G., Soderstrom, K. and Karlstrom, A., 1994. Inactivation of thrombin by a complex between rat mast cell protease-1 and heparin proteoglycan. Biochemical Journal, **299**: 507-513.

Pejler, G. and Berg, L., 1995. Regulation of rat mast cell protease-I activity. Protease inhibition is prevented by heparin proteoglycan. European Journal of Biochemistry, **233**: 192-199.

Pemberton, A.D., Huntley, J.F., Miller, H.R.P., 1996. Sheep mast cell proteinase, a chymase from ovine mucosal mast cells with an unusual trypsin-like activity: characterisation of its native inhibitors. Journal of Biological Chemistry, *submitted.*

Petersen, L.J., Poulsen, L.K., Sondergaard, J. and Skov, P.S., 1994. The use of cutaneous microdialysis to measure substance P-induced histamine release in intact human skin *in vivo.* Journal of Allergy and Clinical Immunology, **94**: 773-783.

Peterson, M.W., Stone, P. and Shasby, D.M., 1987. Cationic neutrophil proteins increase transendothelial albumin movement. Journal of Applied Physiology, **62**: 1521-1530.

Piguet, P.F., 1993. TNF and the pathology of the skin. Research in Immunology, **144**: 320-326.

Pincelli, C., Fantini, F., Magnoni, C. and Giannetti, A., 1994. Psoriasis and the nervous system. Acta Dermato-Venereologica, **74**: S186, S16, 60-61.

Plaut, M., Pierce, J.H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P. and Paul, W.E., 1989. Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. Nature, **339**: 64-67.

Pober, J.S., 1988. Cytokine-mediated activation of vascular endothelium. Physiology and pathology. American Journal of Pathology, **133**: 426-433.

Pober, J.S., Bevilacqua, M.P., Mendrick, D.L., Lapierre, L.A., Fiers, W. and Gimbrone, M.A., 1986. Two distinct monokines interleukin-1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. Journal of Immunology, **136**: 1680-1687.

Powell, M.B., Weisbroth, S.H., Roth, L. and Wilhelmsen, C., 1980. Reaginic hypersensitivity in *Otodectes cynotis* infestation of cats and mode of mite feeding. American Journal of Veterinary Research, **41**: 877-882.

Powers, J.C., Tanaka, T., Harper, J.W., Minematsu, Y., Barker, L., Lincoln, D., Crumley, K.V., Fräki, J.E., Schechter, N.M., Lazarus, G.G., Nakajima, K., Nakashino, K., Neurath, H. and Woodbury, R.G., 1985. Mammalian chymotrypsin-like enzymes. Comparative reactivities of rat mast cell proteases, human and dog skin chymases, and human cathepsin G with peptide 4-nitroanilide substrates and with peptide chloromethylketone and sulphonyl fluoride inhibitors. Biochemistry, **24**: 2048-2058.

Preito, J., Beatty, P.G., Clark, E.A., Patarroyo, M., 1988. Molecules mediating adhesion of T-cell and B-cell, monocytes and granulocytes to vascular endothelial cells. Immunology, **63**: 631-637.

Punnonen, J., Aversa, G., Cocks, B.G., M^{c}Kenzie, A.N., Menon, S., Zurawski, G., De Waal Malefyt, R. and de Vries, J.E., 1993. Interleukin-13 induces interleukin-4 independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proceedings of the National Academy of Sciences of the USA, **90**: 3730-3734.

Qu, Z., Liebler, J.M., Powers, M.R., Galey, T., Ahmadi, P., Huang, X-N., Ansel, J.C., Butterfield, J.H., Planck, S.R. and Rosenbaum, J.T., 1995. Mast cells are a major source of basic fibroblast growth factor in chronic inflammation and cutaneous hemangioma. American Journal of Pathology, **147**: 564-573.

Ramos, B.F., Zhang, Y. and Jakschik, B.A., 1994. Neutrophil elicitation in the reverse passive Arthus reaction - complement-dependent and complemnent-independent mast cell involvement. Journal of Immunology, **152**: 1380-1384.

Razin, E., Cordon-Cardo, C. and Good, R.A., 1981. Growth of a pure population of mouse mast cells *in vitro* with conditioned medium derived from concanavalin A-stimulated splenocytes. Proceedings of the National Academy of Sciences of the USA, **78**: 2559-2561.

Razin, E., Mencia-Huerta, J.M., Lewis, R.A., Corey, E.A. and Austen, K.F., 1982. Generation of leukotriene C_4 from a subclass of mast cells differentiated *in vitro* from mouse bone marrow. Proceedings of the National Academy of Sciences of the USA, **79**: 4665-4667.

Razin, E., Mencia-Huerta, J.M., Stevens, R.L., Lewis, R.A., Liu, F.T, Corey, E.J., and Austen, K.F., 1983. IgE-mediated release of leukotriene C_4, chondroitin sulfate E proteoglycan, β-hexosaminidase, and histamine from cultured bone marrow-derived mouse mast cells. Journal of Experimental Medicine, **157**: 189-201.

Read, G.W., Hing, S.M. and Kiefer, E.F., 1982. Competative inhibition of 48/80-induced histamine release by benzalkonium chloride and its analog and the polyamide receptor in mast cells. Journal of Pharmacology and Experimental Therapeutics, **222**: 652-657.

Regoli, D., Bondon, A. and Fanchére, J-L., 1994. Receptors and antagonists for substance P and related peptides. Pharmacological Reviews, **46**: 551-559.

Reilly, C.F., Tewksbury, D.A., Schechter, N.B. and Travis, J., 1982. Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. Journal of Biological Chemistry, **257**: 8619-8622.

Reilly, D.F., Schechter, N.B. and Travis, J., 1985. Inactivation of bradykinin and kallidin by cathepsin G and mast cell chymase. Biochemical and Biophysical Research Communications, **127**: 443-449.

Renauld, J-C., Kermouni, A., Vink, A., Louahed, J. and Von Snick, J., 1995. Interleukin-9 and its receptor: involvement in mast cell differentiation and T cell oncogenesis. Journal of Leukocyte Biology, **57**: 353-360.

Rennick, D.M., Lee, F.D., Yokota, T., Arai, K.-I., Cantor, H. and Nabel, G.J., 1985. A cloned MCGF cDNA encodes a multilineage haematopoietic growth factor: multiple activities of interleukin 3. Journal of Immunology, **134**: 910-914.

Rennick, D., Hunte, B., Holland, G. and Thompson-Snipes, L-A., 1995. Cofactors are essential for stem cell factor-dependent growth and maturation of mast cell progenitors: comparative effects of interleukin-3 (IL-3), IL-4, IL-10, and fibroblasts. Blood, **85**: 57-65.

Revtyak, G.E., Hughes, M.J., Johnson, A.R., Campbell, W.B., 1988. Histamine stimulation of prostaglandin and HETE synthesis in human endothelial cells.
American Journal of Physiology, **255**: C214-C225.

Reynolds, D.S., Stevens, R.L., Lane, W.S., Carr, M.H., Austen, K.F. and Serafin, W.E., 1990. Different mouse mast cell populations express various combinations of at least six distinct mast cell serine proteases.
Proceedings of the National Academy of Sciences of the USA, **87**: 3230-3234.

Reynolds, D.S., Gurley, D.S., Austen, K.F. and Serafin, W.E., 1991. Cloning of the cDNA and gene of mouse mast cell protease-6. Transcription by progenitor mast cells and mast cells of the connective tissue subclass. Journal of Biological Chemistry, **266**: 3847-3853.

Reynolds, D., Austen, K.F. and Serafin, W.E., 1992. Molecular charcterization of mouse mast cell secretory granule proteases. In: M.Kaliner and D.Metcalf (Editors), The Mast Cell in Health And Disease, Marcel Dekker, Inc., New York, pp. 247-268.

Riley, J.F., 1959. The Mast Cells. E.&D. Livingstone Ltd., Edinburgh & London.

Riley, J.F. and West, G.B., 1956. Skin histamine. Its location in the tissue mast cells. American Medical Association Archives of Dermatology, **74**: 471-478.

Roberts, L.J., Lewis, R.A., Austen, K.F. and Oates, J.A., 1979. Prostaglandin thromboxone and 12-hydroxy-5,8,10,14-eicosatetraenoic acid production by rat mast cells.
Biochemica et Biophysica Acta, **575**: 185-192.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, V.I., Liotta, L.A., Falanga, V., Kehrl, J.H. and Fauci, A.S., 1986. Transforming growth factor β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro.
Proceedings of the National Academy of Sciences of the USA, **83**: 4167-4171.

Robertson, I. and Greaves, M.W., 1978. Responses of human skin blood vessels to synthetic histamine analogues. British Journal of Clinical Pharmacology, **5**: 319-322.

Rot, A., 1991. Chemotactic potency of recombinant human neutrophil attractant/activation protein-1 (interleukin-8) for polymorphonuclear leukocytes of different species.
Cytokine, **3**: 21-27.

Rottem, M., Goff, J.P., Albert, J.P. and Metcalfe, D.D., 1993. The effects of stem cell factor on the ultrastructure of Fc epsilon RI+ cells developing in IL-3-dependent murine bone marrow-derived cell cultures. Journal of Immunology, **151**: 4950-4963.

Rubenstein, I., Nadel, J.A., Graf, P.D. and Caughey, G.H., 1990. Mast cell chymase potentiates histamine-induced wheal formation in the skin of ragweed-allergic dogs.
Journal of Clinical Investigation, **86**: 555-559.

Ruck, P., Horny, H.P. and Kaiserling, E., 1990. Immunoreactivity of human tissue mast cells: nonspecific binding of primary antibodies against regulatory peptides by ionic linkage.
Journal of Histochemistry and Cytochemistry, **38**: 859-867.

Rüger, B., Dunbar, P.R., Hasan, Q., Sawada, H., Kittelberger, R., Greenhill, N. and Neale, T.J., 1994. Human mast cells produce type VIII collagen *in vivo*. International Journal of Experimental Pathology, **75**: 397-404.

Ruitenberg, E.J. and Elgersma, A., 1976. Absence of intestinal mast cell response in congenitally athymic mice during *Trichinella spiralis* infection. Nature, **264**: 258-260.

Ruoss, S.J., Hartmann, T. and Caughey, G.H., 1991. Mast cell tryptase is a mitogen for cultured fibroblasts. Journal of Clinical Investigation, **88**: 493-499.

Sage, H., Woodbury, R.G. and Bornstein, P., 1979. Structural studies of human type IV collagen. Journal of Biological Chemistry, **254**: 9893-9900.

Santos, A.A.D. and Machado, C.R.S., 1994. Histochemical and ultrastructural studies of mast cells in the intestinal mucosa and skin of the opossum *Didelphis albiventris*. Histochemical Journal, **26**: 233-238.

Sayama, S., Iozzo, R.V., Lazarus, G.S. and Schechter, N.M., 1987. Human skin chymotrypsin-like proteinase chymase. Subcellular localization to mast cell granules and interaction with heparin and other glycosaminoglycans. Journal of Biological Chemistry, **262**: 6808-6815.

Schall, T.J. and Bacon, K.B., 1994. Chemokines, leukocyte trafficking, and inflammation. Current Opinion in Immunology, **6**: 865-873.

Schechter, N.M., 1989. Structure of the dermal-epidermal junction and potential mechanisms for its degradation: the possible role of inflammatory cells. Immunology Series, **46**: 477-507.

Schechter, N.M., 1995. Chymotrypsin-like proteinases of human skin mast cells. In: G.H.Caughey (Editor), Mast Cell Proteases in Immunology and Biology, Marcel Dekker Inc., New York, pp. 47-69.

Schechter, N.M., Fräki, J.E., Geesin, J.C. and Lazarus, G.S., 1983. Human skin chymotryptic protease. Isolation and relation to cathepsin G and rat mast cell proteinase I. Journal of Biological Chemistry, **258**: 2973-2978.

Schechter, N.M., Choi, J.K., Slavin, D.A., Deresienski, D.T., Sayama, S., Dong, G., Lavker, R.M., Proud, D. and Lazarus, G.S., 1986. Identification of a chymotrypsin-like proteinase in human mast cells. Journal of Immunology, **137**: 962-970.

Schechter, N.M., Slavin, D., Fetter, R.D., Lazarus, G.S., Fräki, J.E., 1988. Purification and identification of two serine class proteases from dog mast cells biochemically and immunologically similar to human proteinases tryptase and chymase. Archives of Biochemistry and Biophysics, **262**: 232-244.

Schechter, N.M., Spraws, J.L., Schoenberger, O.L., Lazarus, G.S., Cooperman, B.S. and Rubin, H., 1989. Reaction of human skin chymotrypsin-like protease chymase with plasma proteinase inhibitors. Journal of Biological Chemistry, **264**: 21308-21315.

Schick, B., Austen, K.F. and Schwartz, L.B., 1984. Activation of rat serosal mast cells by chymase, an endogenous secretory granule protease. Journal of Immunology, **132**: 2571-2577.

Schick, B. and Austen, K.F., 1986. Rat serosal mast cell degranulation mediated by chymase, an endogenous secretory granule protease: active site-dependent initiation at 1° C.
Journal of Immunology, **136**: 3812-3818.

Schrader, J.W., Lewis, S.J., Clark-Lewis, I. and Culvenor, J.G., 1981. The persisting (p) cell: histamine content, regulation by a T cell-derived factor, origin from a bone marrow precursor and relationship to mast cells.
Proceedings of the National Academy of Sciences of the USA, **78**: 323-327.

Schröder, J-M., 1995. Cytokine networks in the skin.
Journal of Investigative Dermatology, **105**: 20S-24S.

Schwartz, L.B., 1985. Monoclonal antibodies against human mast cell tryptase demonstrates shared antigenic sites on subunits of tryptase and selective localization of the enzyme to mast cells. Journal of Immunology, **134**: 526-531.

Schwartz, L.B., 1989. Heterogeneity of mast cells in humans. In: Galli,S.J. and Austen, K.F. (Editors), Mast Cell and Basophil Differentiation and Function in Health and Disease, Raven Press, New York, pp. 93-106.

Schwartz, L.B., 1994. Mast cells: function and contents.
Current Opinion in Immunology, **6**: 91-97.

Schwartz, L.B., 1995. Mast cell tryptase: properties and roles in human allergic responses. In: G.H.Caughey (Editor), Mast Cell Proteases in Immunology and Biology, Marcel Dekker, Inc., New York, pp. 9-23.

Schwartz, L.B., Austen, K.F. and Wasserman, S.L., 1979. Immunologic release of β-hexosaminidase and β-gluconidase from purified rat serosal mast cells.
Journal of Immunology, **123**: 1445-1450.

Schwartz, L.B., Lewis, R.A. and Austen, K.F., 1981. Tryptase from human pulmonary mast cells: purification and characterization. Journal of Biological Chemistry, **256**: 1939-1943.

Schwartz, L.B., Riedel, C., Caulfield, J.P., Wasserman, S.I. and Austen, K.F., 1981a. Cell association of complexes of chymase, heparin proteoglycan, and protein after degranulation by rat mast cells. Journal of Immunology, **126**: 2071-2078.

Schwartz, L.B., Lewis, R.A., Seldin, D. and Austen, K.F., 1981b. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells.
Journal of Immunology, **126**: 1290-1294.

Schwartz, L.B., Kawahara, M.S., Hugli, T.E., Vik, D., Fearon, D.T. and Austen, K.F., 1983. Generation of C3a anaphylatoxin from human C3 by human mast cell tryptase.
Journal of Immunology, **130**: 1891-1895.

Schwartz, L.B., Bradford, T.R., Littman, B.H. and Wintroub, B.U., 1985. The fibrinogenolytic activity of purified tryptase from human lung mast cells.
Journal of Immunology, **135**: 2762-2767.

Schwartz, L.B. and Bradford, T.R., 1986. Regulation of tryptase from human lung mast cells by heparin. Stabilization of the active tetramer.
Journal of Biological Chemistry, **261**: 7372-7379.

Schwartz, L.B., Irani, A.A., Roller, K., Castells, M.C. and Schechter, N.M., 1987. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. Journal of Immunology, **138**: 2611-2615.

Scott, D.W., 1981. Observations on canine atopy. Journal of the American Animal Hospital Association, **17**: 91-100.

Scott, D.W., 1988. Large Animal Dermatology, 1st Edition. W.B.Saunders Company, Philadelphia, pp. 284-333.

Scott, D.W., 1990. Epidermal mast cells in the cat. Veterinary Dermatology, **1**: 65-69.

Scott, D.W., Smith, M.C. and Manning, T.O., 1984. Caprine dermatology. Part 1. Normal skin and bacterial and fungal disorders. The Compendium on Continuing Education for the Practising Veterinarian, **6**: 5190-5211.

Scott, D.W. and Campbell, S.G., 1987. A seasonal pruritic dermatitis resembling atopy in sheep. Agricultural Practice, **8**: 46-49.

Scott, D.W., Miller, W.H. and Griffin, C.E., 1995. In: Muller & Kirk's Small Animal Dermatology 5th Edition. W.B.Saunders Company, Philadelphia, pp. 31-32, 118, 536-549.

Scudamore, C.L., Pennington, A.M., Thornton, E., M^{c}Millan, L., Newlands, G.F.J. and Miller, H.R.P., 1995. Basal secretion and anaphylactic release of rat mast cell protease-II (RMCP-II) from *ex-vivo* perfused rat jejunum - translocation of RMCP-II into the gut lumen and its relation to mucosal histology. Gut, **37**: 235-241.

Seder, R.A., Paul, W.E., Davis, M.M. and Fazekas de St.Groth, B., 1992. The presence of interleukin-4 during in vitro priming determines the lymphokine producing potential of CD4^{+} T cells from T cell receptor transgenic mice. Journal of Experimental Medicine, **176**: 1091-1098.

Seder, R.A., Gazzinelli, R., Sher, A. and Paul, W.E., 1993. Interleukin-12 acts directly on CD4^{+} T cells to enhance priming for IFNγ production and diminishes IL-4 inhibition of such priming. Proceedings of the National Academy of Sciences of the USA, **90**: 10188-10192.

Selvan, R.S., Butterfield, J.H. and Krangel, S., 1994. Expression of multiple chemokine genes by a human mast cell leukemia. Journal of Biological Chemistry, **269**: 13893-13898.

Seow, H-F., Yoshimura, T., Wood, P.R. and Colditz, I.G., 1994. Cloning, sequencing, expression and inflammatory activity in skin of ovine interleukin-8. Immunology and Cell Biology, **72**: 398-405.

Seppa, H., 1980. The role of chymotrypsin-like protease of the rat mast cells in inflammatory vasopermeability and fibrinolysis. Inflammation, **4**: 1.

Seppa, H.K., Vaananen, K. and Korhonen, K., 1979. Effects of mast cell chymase of rat skin on intercellular matrix: a histochemical study. Acta Histochemica, **64**: 64-70.

Slavin, D.A., Fetter, R.D., Fräki, J.E., Lazarus, G.S. and Schechter, N.M., 1987. Identification of two serine proteinases in dog skin analogous to human skin mast cell proteinases tryptase and chymase. Clinical Research, **35**: no.3, p..A 718.

Smith, C. and Rennick, D., 1986. Characterization of a murine lymphokine distinct from interleukin 2 and interleukin 3 (IL-3) possessing a T-cell growth factor activity and a mast cell growth factor activity that synergizes with IL-3.
Proceedings of the National Academy of Sciences of the USA, **83**: 1857-1861.

Smith, C.H., Soh, C. and Lee, T.H., 1992. Cutaneous histamine metabolism in chronic urticaria. Journal of Allergy and Clinical Immunology, **89**: 944-950.

Smith, J.B., M^{c}Intosh, G.H. and Morris, B., 1970. The traffic of cells through tissues: a study of peripheral lymph in sheep. Journal of Anatomy, **107**: 87-100.

Smith, W.D., Jackson, F., Jackson, E, Williams, J. and Miller, H.R.P., 1984. Manifestations of resistance to ovine ostertagiasis associated with immunological responses in the gastric lymph. Journal of Comparative Pathology, **94**: 591-601.

Sommerhoff, C.P., 1995. Dog mast cell proteinases in models of airway secretion, bronchoconstriction, cutaneous vascular permeability, and tissue fibrosis. In: G.H.Caughey (Editor), Mast Cell Proteases in Immunology and Biology, Marcel Dekker Inc., New York, pp. 145-167.

Sommerhoff, C.P., Ruoss, S.J. and Caughey, G.H., 1992. Mast cell proteoglycans modulate the secretagogue, proteoglycanase and amidolytic activities of dog mast cell chymase. Journal of Immunology, **148**: 2859-2866.

Sonoda, T., Ohno, T. and Kitamura, Y., 1982. Concentration of mast cell progenitors in bone marrow, spleen and blood of mice determined by limiting dilution analysis.
Journal of Cellular Physiology, **112**: 136-140.

Soter, N.A., Lewis, R.A., Corey, E.J. and Austen, K.F., 1983. Local effects of synthetic leukotrienes (LTC_4, LTD_4, LTE_4 and LTB_4) in human skin.
Journal of Investigative Dermatology, **80**: 115-120.

Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. Cell, **76**: 301-314.

Springman, E.B. and Serafin, W.E., 1995. Secretory endo- and exopeptidases of mouse mast cells: structure, genetics, and regulation of expression. In: G.H.Caughey (Editor), Mast Cell Proteases In Immunology and Biology, Marcel Dekker, Inc., New York, pp.169-201.

Sredni, B., Friedman, M.M., Bland, C.E. and Metcalfe, D.D., 1983. Ultrastructural, biochemical, and functional characteristics of histamine-containing cells cloned from mouse bone marrow : tentative identification as mucosal mast cells.
Journal of Immunology, **131**: 915-922.

Staemmler, M. (1921) Untersuchung über vorkommen und bedentung der histiogenen mastzellen im menschlichen körper unter normalen und pathologischen verhältnissen.
Frankf. Ztscher. Pathol., **25**: 391.

Stevens, R.L., Katz, H.R., Seldin, D.C. and Austen, K.F., 1986a. Biochemical characteristics distinguish subclasses of mammalian mast cells. In: A.D.Befus et al (Editors), Mast Cell Differentiation and Heterogeneity, Raven Press, New York, pp. 183-203.

Stevens, R.L., Lee, T.D.G., Seldin, O.C., Austen, K.F., Befus, A.D. and Bienenstock, J., 1986b. Intestinal mucosal mast cells from rats infected with Nippostrongylus brasiliensis contain protease-resistant chondroitin sulphate di-B proteoglycans.
Journal of Immunology, **137**: 291-295.

Stevens, R.L., Fox, C.C., Lichtenstein, L.M. and Austen, K.F., 1988. Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells.
Proceedings of the National Academy of Sciences of the USA, **85**: 2284-2287.

Stevens, R.L. and Austen, K.F., 1989. Recent advances in the cellular and molecular biology of mast cells. Immunology Today, **10**: 381-386.

Stevens, R.L., Friend, D.S., M^{c}Neil, H.P., Schiller, V., Ghildyal, N. and Austen, K.F., 1994. Strain-specific and tissue-specific expression of mouse mast cell secretory granule proteases.
Proceedings of the National Academy of Sciences of the USA., **91**: 128-132.

Sture, G.H., Halliwell, R.E.W., Thoday, K.L., Van den Broek, A.H.M., Henfrey, J.I., Lloyd, D.H., Mason, I.S. and Ferguson, E., 1995. Canine atopic disease: the prevalence of positive intradermal skin tests at two sites in the north and south of Great Britain.
Veterinary Immunology and Immunopathology, **44**: 293-308.

Swensson, O., Shubert, C., Christophers, E. and Schröder, J-M., 1991. Inflammatory properties of neutrophil-activating protein-1/interleukin 8 (NAP-1/IL-8) in human skin: a light- and electronmicroscopic study. Journal of Investigative Dermatology, **96**: 682-689.

Tainsh, K.R., Lau, H.Y.A., Liu, W.L. and Pearce, F.L., 1991. The human skin mast cell: a comparison with the human lung cell and a novel mast cell type, the uterine mast cell.
Agents and Actions, **33**: 16-19.

Tainsh, K.R. and Pearce, F.L., 1992. Mast cell heterogeneity: evidence that mast cells isolated from various connective tissue locations in the rat display markedly graded phenotypes. International Archives of Allergy and Applied Immunology, **98**: 26-34.

Tainsh, K.R., Liu, W.L., Lau, H.Y., Cohen, J. and Pearce, F.L., 1992. Mast cell heterogeneity in man - unique functional properties of skin mast cells in response to a range of polycationic stimuli. Immunopharmacology, **24**: 171-180.

Takeda, K., Hatamochi, A. and Ueki, H., 1989. Increased number of mast cells accompany enhanced collagen synthesis in linear localized scleroderma.
Archives of Dermatological Research, **281**: 288-290.

Talukdar, A.H., Calhoun, M.L. and Stinson, A.W., 1972. Microscopic anatomy of the skin of the horse. American Journal of Veterinary Research, **33**: 2365-2390.

Tam, E.K., 1995. Mast cell neuropeptidases. In: G.H. Caughey (Editor), Mast Cell Proteases in Immunology and Biology. Marcel Dekker Inc., New York, pp. 71-107.

Tam, E.K. and Caughey, G.H., 1990. Degradation of airway neuropeptides by human lung tryptase. American Journal of Respiratory Cellular and Molecular Biology, **3**: 27-32.

Tas, J. and Berndsen, R.G., 1977. Does heparin occur in mucosal mast cells of the rat small intestine? Journal of Histochemistry and Cytochemistry, **25**: 1058-1062.

Thompson, R.G., 1978. Inflammation and Repair. In: General Veterinary Pathology. W.B. Saunders Company, Philadelphia, pp. 152-261.

Thompson-Snipes, L., Dhar, V., Bond, M.W., Mosmann, T.R., Moore, K.W. and Rennick, D.M., 1991. Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. Journal of Experimental Medicine, **173**: 507-510.

Tomoe, S., Iwamoto, I., Tomioka, H. and Yoshida, S., 1992. Comparison of substance P-induced and compound 48/80-induced neutrophil infiltrations in mouse skin. International Archives of Allergy and Immunology, **97**: 237-242.

Torii, I., Morikawa, S., Harada, T. and Kitamura, Y., 1993. Two distinct types of cellular mechanisms in the development of delayed hypersensitivity in mice: requirement of either mast cells or macrophages for elicitation of the response. Immunology, **78**: 482-490.

Tsai, M., Takeishi, T., Thompson, H., Langley, K., Zsebo, K., Metcalf, D., Geissler, E. and Galli, S.J., 1991a. Induction of mast cell proliferation, maturation and heparin synthesis by the rat c-kit ligand, stem cell factor.
Proceedings of the National Academy of Sciences of the USA, **88**: 6382-6386.

Tsai, M., Shih, L-S., Newlands, G.F.J., Takeishi, T., Langley, K.E., Zsebo, K.M., Miller, H.R.P., Geissler, E.N. and Galli, S.J., 1991b. The rat *c-kit* ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells in vivo. Analysis by anatomical distribution, histochemistry, and protease phenotype.
Journal of Experimental Medicine, **174**: 125-131.

Unna, P.G., 1887. Dermatologische studien Beiträge zur anatomie und pathogenese der urticaria simplex und pigmentosa. Zur kenntnis des elastischen gewebes der haut. Leopold Voss, Hamburg & Leipzig.

Unna, P.G., 1894. Histopathologie der hautkrankheiten. (Neurofibrom, urticaria pigmentosa, miliaria, syphilidie). Leopold Voss, Hamburg & Leipzig.

Urata, H., Kinoshita, A., Misono, K.S., Bumpus, F.M. and Husain, A., 1990. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. Journal of Biological Chemistry, **265**: 22348-22357.

Valent, P., Schmidt, G., Besmer, J., Mayer, P., Zenke, G., Liehl, E., Hinterberger, W., Lechner, K., Maurer, D. and Bettelheim, P., 1989. Interleukin-3 is a differentiation factor for human basophils. Blood, **73**: 1763-1769.

Valent, P., Besmer, J., Sillaber, C.H., Butterfield, J.H., Eher, R., Majdic, O., Kishi, K., Klepetko, W., Eckersberger, F., Kaltenbrunner, R., Lechner, K. and Bettelheim, P., 1990. Failure to detect IL-3 binding sites on human mast cells.
Journal of Immunology, **145**: 3432-3437.

Valent, P., Spanblöchel, E., Sperr, W.R., Sillaber, C., Zsebo, K.M., Agis, H., Strobl, H., Geissler, K., Bettelheim, P. and Lechner, K., 1992. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long term culture Blood, **80**: 2237-2245.

Vallone, A., 1944. 'Le mastzellen' dei connettivi nei diversi organi e nei vari processi morbosi dell'uomo. Arch. De Vecchi Anat.Pat., **7**: 249.

Van Loveren, H., Teppema, J.S. and Askenase, P.W., 1990. Skin mast cells. In: J.D.Bos (Editor), Skin Immune System. CRC Press Inc., Florida, pp. 171-193.

Vanderslice, P., Ballinger, S.M., Tam, E.K., Goldstein, S.M., Craik, C.S. and Caughey, G.H., 1990. Human mast cell tryptase: multiple cDNAs and genes reveal a multi-gene serine protease family.
Proceedings of the National Academy of Sciences of the USA., **87**: 3811-3815.

Vegad, J.L., 1970. Staining of mast cells and eosinophiles in the sheep skin.
New Zealand Veterinary Journal, **18**: 31-33.

Veil, C., 1957. Contribution à la physiologie des labrocytes des ècailles de poisson mise en evidence de réactions anaphylactiques et comportement en présence d´un libèrateur d´histamine. Acta Physiologica et Pharmacologica, **6**: 386.

Vercelli, D., Jabara, H.H., Arai, K.I., Yokota, T. and Geha, R.S., 1989. Endogenous interleukin-6 plays an obligatory role in interleukin-4-dependent human IgE synthesis.
European Journal of Immunology, **19**: 1419-1424.

Volc-Platzer, B., Valent, P., Radaszkiewicz, T., Mayer, P., Bettelheim, P. and Wolff, K., 1991. Recombinant human interleukin 3 induces proliferation of inflammatory cells and keratinocytes *in vivo*. Laboratory Investigation, **64**: 557-566.

Von Recklinghausen, F., 1863. Über eiter und bindgewebskörperchen.
Virchows Archiv., **28**: 157.

Wahlgren, C.F., Hagermark, O. and Bergstrom, R., 1991. Patients perception of itch induced by histamine, compound 48/80 and wool fibres in atopic dermatitis. Acta Dermato-Venereologica (Stockholm), **71**: 488-494.

Walls, A.F., 1995. The roles of neutral proteases in asthma and rhinitis. In: W.W.Busse and S.T.Holgate (Editors), Asthma and Rhinitis. Blackwell Scientific Publications, Oxford, pp. 801-824.

Walls, A.F., Brain, S.D., Desai, A., Jose, P.J., Hawkins, E., Church, M.K. and Williams, T.J., 1992. Human mast cell tryptase attenuates the vasodilator activity of calcitonin gene-related peptide. Biochemical Pharmacology, **43**: 1243-1248.

Walls, A.F., He, S., Teran, L. and Holgate, S.T., 1993. Mast cell proteases as mediators of vascular leakage and cell accumulation.
Journal of Allergy and Clinical Immunology, **91**: 256.

Walls, A.F., He, S., Teran, L., Zhang, L. and Holgate, S.T., 1994. Induction of neutrophil accumulation by mast cell proteases. Clinical and Experimental Allergy, **24**: 191.

Walsh, L.J., 1995. Ultraviolet irradiation of skin induces mast cell degranulation and release of tumour necrosis factor-α. Immunology and Cell Biology, **73**: 226-233.

Walsh, L.J., Lavker, R.M. and Murphy, G.F. 1990. Determinants of immune cell trafficking in the skin. Laboratory Investigation, **63**: 592-600.

Walsh, L.J., Trinchieri, G., Waldorf, H.A., Whitaker, D. and Murphy, G.F., 1991. Human dermal mast cells contain and release tumor necrosis factor α, which induces endothelial leukocyte adhesion molecule 1.
Proceedings of the National Academy of Sciences of the USA., **88**: 4220-4224.

Wawryk, S.O., Novotny, J.R., Wicks, I.P., Wilkinson, D., Maher, D., Salvaris, E., Welch, K., Fecondo, J. and Boyd, A.W., 1989. The role of the LFA-1/ICAM-1 interaction in human leukocyte homing and adhesion. Immunological Reviews, **108**: 135-161.

Wedmore, C.V. and Williams, T.J., 1981. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature, **289**: 646-650.

Weidner, N. and Austen, K.F., 1993. Heterogeneity of mast cells at multiple body sites: fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase, and carboxypeptidase content. Pathology Research and Practice, **189**: 156-162.

Weisbroth, S.H., Powell, M.B., Roth, L. and Scher, S., 1974. Immunopathology of naturally occurring otodectic otoacariasis in the domestic cat.
Journal of the American Veterinary Medical Association, **165**: 1088-1093.

Weiss, R.R., Whitaker-Menezes, D., Longley, J., Bender, J. and Murphy, G.F., 1995. Human dermal endothelial cells express membrane-associated mast cell growth factor.
Journal of Investigative Dermatology, **104**: 101-106.

Welle, M.M., Proske, S.M., Harvima, I.T., Schechter, N.M., 1995. Demonstration of tryptase in bovine cutaneous and tumor mast cells.
The Journal of Histochemistry and Cytochemistry, **43**: 1139-1144.

Wershil, B.K., Mekori, Y.A., Murakami, T. and Galli, S.J., 1987. ^{125}I-fibrin deposition in IgE-dependent immediate hypersensitivity reactions in mouse skin. Demonstration of the role of mast cells using genetically mast cell-deficient mice locally reconstituted with cultured mast cells. Journal of Immunology, **139**: 2605-2614.

Wershil, B.K., Murakami, T. and Galli, S.J., 1988. Mast cell-dependent amplification of an immunologically nonspecific inflammatory response. Mast cells are required for the full expression of cutaneous acute inflammation induced by phorbol 12-myristate 13-acetate.
Journal of Immunology, **140**: 2356-2360.

Wershil, B.K., Wang, Z-S., Gordon, J.R. and Galli, S.J., 1991. Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor-alpha.
Journal of Clinical Investigation, **87**: 446-453.

Wershil, B.K., Furuta, G.T., Lavigne, J.A., Choudhury, A.R., Wang, Z-S. and Galli, S.J., 1995. Dexamethasone or cyclosporin A suppress mast cell-leukocyte cytokine cascades. Multiple mechanisms of IgE- and mast cell-dependent cutaneous inflammation in the mouse.
Journal of Immunology, **154**: 1391-1398.

White, M.A., 1990. The role of histamine in allergic diseases.
Journal of Allergy and Clinical Immunology, **86**: 599-605.

White, M.V., Slater, J.E. and Kaliner, M.A., 1987. Histamine and asthma. American Review of Respiratory Disease, **135**: 1165-1176.

Wiedow, O., Schröder, J.M., Gregory, H., Young, J.A. and Christophers, E., 1990. Elafin: an elastase-specific inhibitor of human skin: purification, characterization, and complete amino acid sequence. Journal of Biological Chemistry, **265**: 14791-14795.

Wiesner-Menzel, L., Schulz, B., Vakilzadeh, F. and Czarnetzki, B.M., 1981. Electron microscopical evidence for a direct contact between nerve fibres and mast cells. Acta Dermatovener (Stockholm), **61**: 465-469.

Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., Burgess, G.S., Cosman, D. and Lyman, S.D., 1990. Identification of a ligand for the *c-kit* proto-oncogene. Cell, **63**: 167-174.

Wintroub, B.U., Schechter, N.B., Lazarus, G.S., Kaempfer, C.E. and Schwartz, L.B., 1984. Angiotensin I conversion by human and rat chymotryptic proteinases. Journal of Investigative Dermatology, **83**: 336-339.

Wintroub, B.U., Kaempfer, C.E., Schechter, N.M. and Proud, D., 1986. A human mast cell chymotrypsin-like enzyme. Identification and partial characterisation. Journal of Clinical Investigation, **77**: 196-201.

Wodnar-Filipowicz, A., Heusser, C.H. and Moroni, C., 1989. Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. Nature, **339**: 150-152.

Woodbury, R.G., Gruzenski, G.M. and Lagunoff, D., 1978. Immunofluorescent localization of a serine protease in rat small intestine. Proceedings of the National Academy of Sciences of the USA., **75**: 2785-2789.

Woodbury, R.G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D. and Neurath,H., 1978. An intracellular serine protease from rat skeletal muscle in the chymotrypsin-like enzymes of mast cells. Proceedings of the National Academy of Sciences of the USA., **75**: 5311-5313.

Yamamoto, S., Nakamura, K., Tanaka, T., Morita, E. and Kameyoshi, Y., 1995. Keratinocyte-derived modulators of mast cell growth. In: Y. Kitamura, S. Yamamoto, S.J. Galli and M.W. Greaves (Editors), Biological and Molecular Aspects of Mast Cell and Basophil Differentiation and Function. Raven Press Ltd., New York, pp. 131-138.

Yano, H., Wershil, B.K., Arizono, N. and Galli, S.J., 1989. Substance P-induced augmentation of cutaneous vascular permeability and granulocyte infiltration in mice is mast cell dependent. Journal of Clinical Investigation, **84**: 1276-1286.

Yezzi, M.J., Hsieh, I.E. and Caughey, G.H., 1994. Mast cell and neutrophil expression of dog mast cell protease-3. A novel tryptase-related serine protease. Journal of Immunology, **152**: 3064-3072.

Yi, E.S. and Ulich, T.R., 1992. Endotoxin, interleukin-1, and tumor necrosis factor cause neutrophil-dependent microvascular leakage in postcapillary venules. American Journal of Pathology, **140**: 659-663.

Yilmaz, H., Haverson, K. and Morgan, K.L., 1994. Obtention of ovine IgE from heterohybridoma. Veterinary Research, **25**: 574-579.

Yirrell, D.L., Reid, H.W., Norval, M. and Miller, H.R.P., 1991. Qualitative and quantitative changes in ovine afferent lymph draining the site of epidermal orf virus infection. Veterinary Dermatology, **2**: 133-141.

Yurt, R. and Austen, K.F., 1977. Preparative purification of the rat mast cell chymase. Characterization and interaction with granule components. The Journal of Experimental Medicine, **146**: 1405-1419.

Zelickson, A.S., 1985. The clinical use of electron microscopy in dermatology. Bolger Publications, Minneapolis.

Zhang, Y., Ramos, B.F. and Jakschik, B.A., 1991. Augmentation of reverse Arthus reaction by mast cells in mice. Journal of Clinical Investigation, **88**: 841-846.

Zhang, Y., Ramos, B.F. and Jakschik, B.A., 1992a. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. Science, **285**: 1957-1959.

Zhang, Y., Ramos, B.F. and Jakschik, B.A., 1992b. Role of mast cells in plasma permeation due to immune injury of the skin basement membrane. Immunology, **77**: 422-427.

Zimmermann, A., 1908. Über das vorkommen der mastzellen beim meerschwinchen. Arch. Mikros.Anat., **72**: 662.

Zsebo, K.M., Wypych, J., M^{c}Niece, I.K., Lu, H.S., Smith, K.A., Karkare, S.B., Sachdev, R.K., Yuschenkoff, V.N., Birkett, N.C., Williams, L.R., Satyagal, V.N., Tung, W., Bosselman, R.A., Mendiaz, E.A. and Langley, K.E., 1990a. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. Cell, **63**: 195-201.

Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R-Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Cattenach, B.M., Galli, S.J. and Suggs, S.V., 1990b. Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the *c-kit* tyrosine kinase receptor. Cell, **63**: 213-224.

APPENDIX A

BUFFERS, SOLUTIONS AND FIXATIVES

A.1. Phosphate-Buffered Saline (PBS)

8g NaCl
0.2 g KCl
1.15g Na_2HPO_4
0.2g KH_2PO_4

Dissolve the above in 1 litre de-ionised, distilled water and adjust pH to 7.2 - 7.3.

A.2. 4% Paraformaldehyde/PBS

8g paraformaldehyde

Dissolve the above in 200ml PBS. Use gentle heat in a fume cupboard and allow the solution to cool before use.

A.3. 5% Bovine Serum Albumin/PBS

5g BSA

Dissolve the above in 100ml PBS.

A.4. 3,3´-diaminobenzidine/PBS Solution

40mg 3,3´-diaminobenzidine tetrahydrochloride

Dissolve the above in 100ml PBS in a fume cupboard. The solution can be divided into 10ml aliquots and frozen at -70 °C until use. Once thawed, and just prior to use, add 20µl H_2O_2 (Hydrogen peroxide solution about 30% ($^{w}/_{v}$) H_2O_2, [100 vols.], BDH, AnalaR Poole, UK, Cat.No. 10128) to 10ml of solution.

A.5. HBSS/Heparin Tissue Culture Medium

Consists of:-

500ml Hanks' Balanced Salt Solution (Gibco BRL, Life Technologies Ltd., Paisley, UK, Cat.No. 041-04020M).

10ml Penicillin/Streptomycin solution (Penicillin-Streptomycin solution, 10000 units penicillin and 10000µg streptomycin per ml, Gibco BRL, Life Technologies Ltd., Paisley, UK, Cat.No. 04305140D).

10000 units heparin (Heparin (tissue culture grade 1A), Sigma, Poole, UK, Cat.No. H3149).

A.6. IMDM/10% FCS Tissue Culture Medium

Consists of:-

500ml Iscove's Modification of Dulbecco's Medium (Gibco BRL, Life Technologies Ltd., Paisley, UK, Cat.No. 041-01980M).

50ml Heat-inactivated Foetal Calf Serum (Advanced Protein Products Ltd., Brierly Hill, UK, Cat.No. AS-302-50; inactivated by heating in a water bath at 56 °C for 30 minutes).

10ml Penicillin/Streptomycin solution (as above).

50µl 2-ME (50mM 2-Mercaptoethanol Gibco BRL, Life Technologies Ltd., Paisley, UK, Cat.No. 043-01350D).

A.7. Coating Buffer For SMCP ELISA

0.1M $NaHCO_3$	(0.84g $NaHCO_3$ in 100ml de-ionised, distilled water).
0.1M Na_2CO_3	(1.06g Na_2CO_3 in 100ml de-ionised, distilled water).

The 0.1M $NaHCO_3$ is titrated to pH 9.6 with the 0.1M Na_2CO_3. The resulting buffer can be stored at -20 °C in 10 ml aliquots until use.

A.8. Citrate/Phosphate Buffer For SMCP ELISA

0.1M Citric acid	24.4ml
0.2M Na_2HPO_4	25.6ml
Distilled water	50.0ml

The pH of the buffer should be 5.0.

A.9. Substrate For SMCP ELISA

100ml Citrate/phosphate buffer, pH 0.5 as above.

40mg Orthophenylenediamine (Sigma Chemical Company Ltd., Poole, UK, Cat.No. P1526)

40µl H_2O_2 (Hydrogen peroxide 30% ($^{w}/_{w}$), [100 vols.], Sigma, Poole, UK, Cat.No. H1009)

The substrate is made immediately prior to use.

A.10. Substrate For Assay Of Arylsulfatase Activity

A.10.1. Acetate Buffer

0.2M Sodium acetate (27.22g Sodium acetate in 1000ml distilled water), titrated to pH 5.7

with concentrated HCL.

A.10.2. Substrate

6.255mM p-nitrocatechol sulphate i.e. 9.75mg p-nitrocatechol sulphate (Sigma Chemical Company Ltd., Poole, UK, Cat.No. N7251) in 5ml of acetate buffer, pH 5.7 (above).

A.11. β-hexosaminidase Assay Buffers And Substrate

A.11.1. Citrate Buffer, pH 4.5

A.11.1.1. Solution A

1N NaOH	300ml
Citric acid	31.5g

The above is made up to 1 litre with de-ionised, double distilled water.

A.11.1.2. Solution B

0.1N HCL

Working citrate buffer solution = 67.8ml solution A plus 32.2ml solution B, titrated to pH 4.5.

A.11.2. Substrate

p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Company Ltd., Poole, UK, Cat.No. N9376). 5mM substrate solution = 1.7mg substrate per ml of citrate buffer, pH 4.5.

A.11.3. Glycine NaOH Stop Solution, pH 10.7

A.11.3.1. Solution A

7.5g glycine
5.8g NaCl

The above is made up to 500ml with de-ionised, double distilled water.

A.11.3.2. Solution B

0.2N NaOH

Working glycine NaOH solution = 52.8ml solution A plus 47.2ml solution B, titrated to pH 10.7.

The glycine NaOH solution should be ice-cold when used.

A.12. Carbol Chromotrope (0.5% Chromotrope In 1% Phenol) Stain

5g Chromotrope (Chromotrope 2R, Gurr® microscopy materials, BDH, Poole, UK, Cat.No. 34020HP).

10g Phenol

The above is dissolved in 1 litre of deionised, distilled water.

APPENDIX B

ADDITIONAL DATA

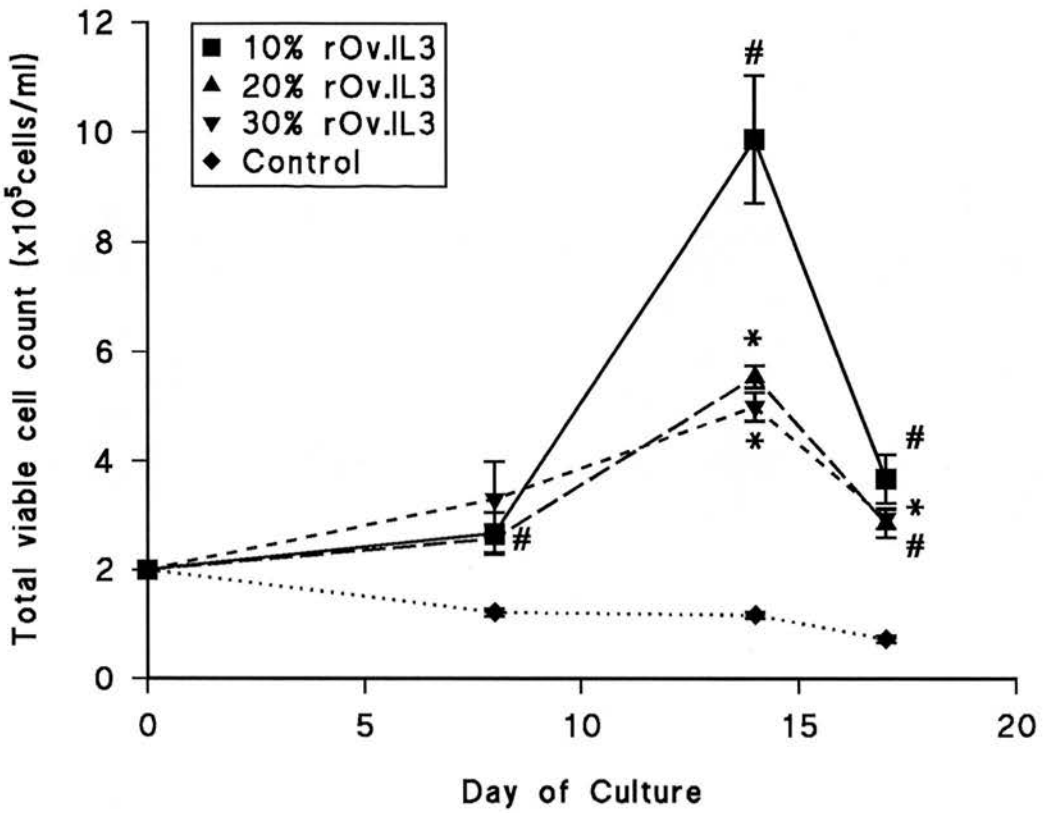


Figure B.1. Growth of bone marrow cells shown as total viable cell count (x10^5 cells/ml) plotted against time (days) for 10%, 20% and 30% rOv.IL-3 (Mean ± SEM; n=3). # $P<0.05$ compared with IMDM/10% FCS control and * represents a similar effect at $P<0.01$. (N.B. # at day 8 refers to 20% rOv.IL-3, * at day 17 refers to 30% rOv.IL-3). Maximal viable cell count was achieved with 10% rOv.IL-3 at day 14 (9.8 ± 1.2 x10^5 cells/ml).

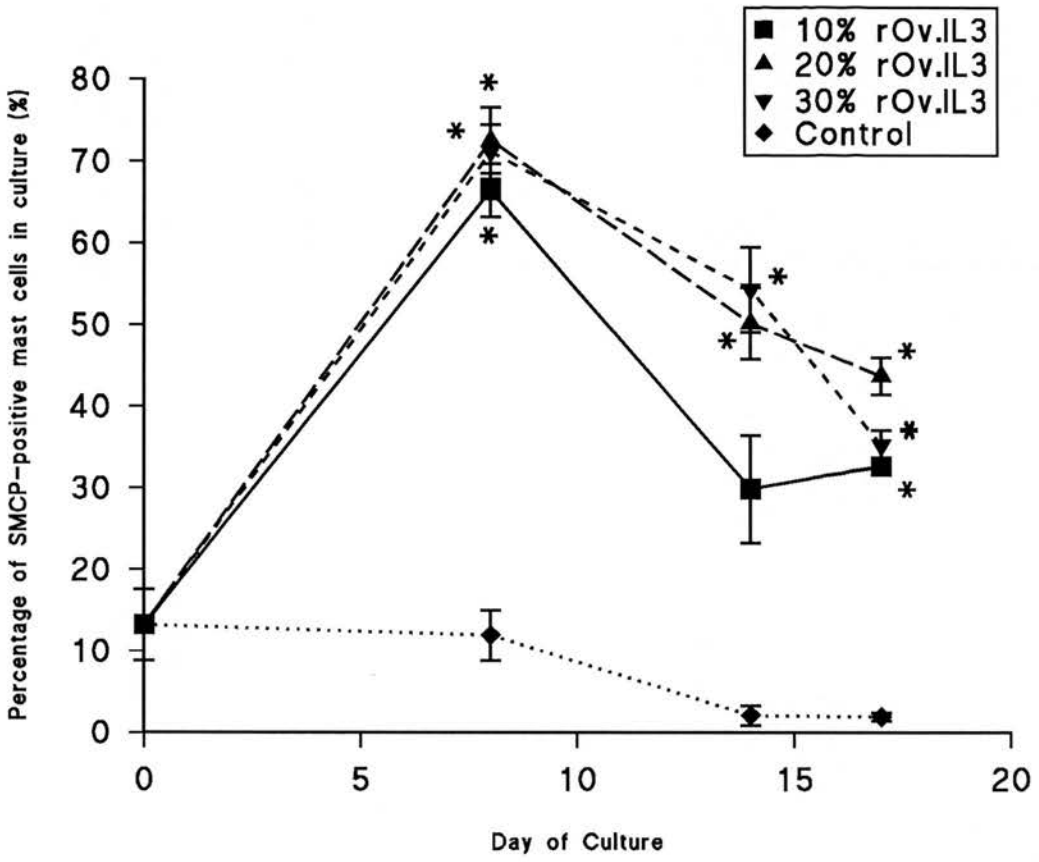


Figure B.2. The effect of rOv.IL-3 concentration on the growth of BMMC. The percentage of SMCP-positive mast cells is plotted against time (days) for 10%, 20% and 30% rOv.IL-3 (Mean ± SEM; n=3). * $P<0.01$ compared with IMDM/10%FCS control.

The mean peak percentage of SMCP-positive mast cells with 10% rOv.IL-3 was 66.4 ± 3.2% at day 8. At day 14, when the total viable cell count is also taken into consideration, the greatest number of BMMC is obtained with 10% rOv.IL-3.

APPENDIX C

PUBLISHED PAPERS

The published paper "Sture, GH., Huntley, JF., MacKellar, A. and Miller, HRP. (1995), Ovine mast cell heterogeneity is defined by the distribution of sheep mast cell proteinase. Veterinary Immunology and Immunopathology **48,** 275-285", can be found in the pocket on the inside of the back cover.

Reprinted from

Veterinary immunology and immunopathology

Veterinary Immunology and Immunopathology
48 (1995) 275–285

Ovine mast cell heterogeneity is defined by the distribution of sheep mast cell proteinase

G.H. Sture [a,*], J.F. Huntley [b], A. MacKellar [b], H.R.P. Miller [a]

[a] *Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Veterinary Field Station, Easter Bush, Nr. Roslin, EH25 9RG, UK*
[b] *Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, UK*

ELSEVIER

Veterinary immunology and immunopathology

an international journal of comparative immunology

Aims and scope. This journal deals with the study of veterinary immunology and immunopathology as applied to domestic animals, laboratory animals and other species that are useful to man.
Within this scheme, manuscripts should deal with subjects such as: basic immunology (fundamental research on the immune system of animals); immunology of spontaneous tumors in animals; research on experimental transplantations; immunochemistry (studies on Ig classes and subclasses of animals, complement and other); new techniques and developments in veterinary immunodiagnosis, immunological aspects of pregnancy and transfer of passive immunity; comparative immunology; immunogenetics, including research on bloodgroups; biological properties of immunoglobulins; clinical immunology in animals; those aspects of immunoprophylaxis, immunology and immunopathology of parasitic and infectious diseases that help in the understanding of immunologic systems. As far as laboratory animals are concerned, very specialized studies that pertain to the understanding of fundamental immunologic principles will in general not be included.
Besides being of interest to those working on relevant aspects of veterinary science, the journal will, because of the comparative nature of much of the material published in this subject field, also be of value to those working in medical immunology and immunopathology.
Circumstances relating to animal experimentation must meet the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for International Organizations of Medical Sciences (obtainable from: Executive Secretary C.I.O.M.S., c/o W.H.O., Via Appia, CH-1211 Geneva 27, Switzerland)

Publication information: *Veterinary Immunology and Immunopathology* (ISSN 0165-2427). For 1995 volumes 43–48 are scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be made within six months of our publication (mailing) date. Please address all your requests regarding orders and subscription queries to: **Elsevier Science B.V., Journal Department, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. Tel: +31 20 4853 642; fax: +31 20 4853 598.**
In the USA and Canada: For further information on this and other Elsevier journals please contact: **Elsevier Science Inc., Journal Information Center, 655 Avenue of the Americas, New York, NY 10010, USA. Tel: (212) 633 3750; fax: (212) 633 3764; telex 420 643 AEP UI.**

Back volumes: Please contact the Publisher.

ELSEVIER

Veterinary Immunology and Immunopathology
48 (1995) 275–285

Veterinary immunology and immunopathology

Ovine mast cell heterogeneity is defined by the distribution of sheep mast cell proteinase

G.H. Sture [a,*], J.F. Huntley [b], A. MacKellar [b], H.R.P. Miller [a]

[a] *Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Veterinary Field Station, Easter Bush, Nr. Roslin, EH25 9RG, UK*
[b] *Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH, UK*

Accepted 8 February 1995

Abstract

The presence or absence of the granule chymase, sheep mast cell proteinase (SMCP), was determined in trachea, bronchus, bronchial lymph node, lung, thymus, spleen, liver, flank skin, abomasum, duodenum, jejunum, ileum, colon and mesenteric lymph node by immunohistochemistry and by enzyme-linked immunosorbent assay using a polyclonal, affinity purified anti-SMCP antibody. Additionally, the presence of putative ovine mast cell subsets was investigated by comparing the number of mast cells identified histochemically (toluidine blue pH 0.5) with the number detected by immunostaining. The thymus had the greatest density of mast cells (225.7 ± 23.4 cells mm^{-2}, histochemically) and the highest concentration of SMCP (19.7 ± 9.3 μg SMCP g^{-1} wet tissue). There was a high degree of correlation between toluidine blue and anti-SMCP cell counts for all tissues ($r^2 = 0.96$, $P < 0.001$) with the exception of skin and liver. On the basis of reactivity to the anti-SMCP antibody, two populations of mast cells were defined, notably those in gastrointestinal tissues (analogous to the mucosal mast cell subset) and those present in skin (the putative ovine connective tissue mast cell subset). Ovine mast cell heterogeneity, resulting from differential expression of SMCP, was thus confirmed.

Keywords: Ovine; Mast cell; Skin; Proteinase; Protease; Heterogeneity

1. Abbreviations

CTMC, connective tissue mast cell; DAB, 3,3′-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; MMC, mucosal mast cell; MMCP, mouse mast cell

* Corresponding author.

SSDI 0165-2427(95)05438-3

proteinase; PBS, phosphate buffered saline; RMCP, rat mast cell proteinase; SMCP, sheep mast cell proteinase.

2. Introduction

Mast cells in rodents and man are a phenotypically heterogeneous cell population with respect to tissue site, fixation properties, histochemical staining, biochemistry and functional activity (reviewed in Galli, 1990). These differences are reflected by their granule constituents, most notably in their content of intragranule proteinases. Chymotrypsin-like mast cell proteinases (chymases) have been isolated from mast cells of the rat (Lagunoff and Pritzl, 1976, Woodbury et al., 1978), mouse (Newlands et al., 1987), man (Schechter et al., 1986) and sheep (Huntley et al., 1986). The elucidation of differences in proteinase expression has led to the identification of mast cell subsets in the rat (Gibson and Miller, 1986; Gibson et al., 1987; Huntley et al., 1990), mouse (Miller et al., 1989) and man (Schwartz, 1989). In the rat and mouse, distinct proteinases are predominantly associated with either the mucosal mast cell (MMC) or connective tissue mast cell (CTMC) subsets. Thus, rat mast cell proteinase-II (RMCP-II) is chiefly present in the gastrointestinal MMC subset, whereas rat mast cell proteinase-I (RMCP-I) is found principally in the CTMC subset. RMCP-I and RMCP-II are the best phenotypic markers available for rat MMC and CTMC, although they do show some overlap (Gibson et al., 1987). In the mouse, mouse mast cell proteinase-1 (MMCP-1) is uniquely present in the MMC subset, whereas mouse mast cell proteinase-4 (MMCP-4) predominates in the CTMC subset (Reynolds et al., 1990). In man, mast cells comprise a population that contains tryptase and chymase (MC^{TC}) and one that contains only tryptase (MC^{T}). Most anatomical sites are believed to contain a mixture of both of these types, although MC^{T} prevails in the lung and small intestinal mucosa, whereas MC^{TC} is chiefly found in skin and small intestinal submucosa (Schwartz, 1989).

Sheep mast cell proteinase (SMCP) has been isolated and characterised as a serine endopeptidase (Knox and Huntley, 1987) and the mast cell source of this enzyme confirmed immunohistochemically (Huntley et al., 1986). SMCP is released locally into lymph and systemically into blood following abomasal challenge with gastric nematodes in immune sheep (Huntley et al., 1987). Whilst the concentration of SMCP in the gastrointestinal mucosa is significantly correlated with mast cell counts, the distribution of SMCP in non-mucosal tissues is unknown. The purpose of this study was, therefore, to compare the distribution of SMCP and of mast cells in a variety of tissues and organs with a view to further defining mast cell heterogeneity in the sheep.

3. Materials and methods

3.1. Animals

Four Suffolk-cross lambs, aged 5–12 months, were studied. These had been grazed at pasture and had not undergone any anthelmintic treatment immediately prior to investi-

gation. At post-mortem examination, samples from trachea, bronchus, bronchial lymph node, lung, thymus, spleen, liver, flank skin, abomasum, duodenum, jejunum, ileum, colon and mesenteric lymph node were taken and processed as described below.

3.2. Preparation of tissue

Tissue for histochemistry and immunohistochemistry was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 for 6 h (Newlands et al., 1984). Fixed samples were then stored in 70% ethanol at 4°C until processing (Miller et al., 1983) after which they were embedded in paraffin wax.

3.3. Histochemical staining

Serial 4 μm sections were stained with toluidine blue pH 0.5 (Enerback, 1966).

3.4. Immunohistochemistry

The standard immunohistochemical technique used was the peroxidase–3,3′-diaminobenzidine (DAB) method outlined below, except in heavily pigmented skin. In order to distinguish immunostaining of SMCP in dermal mast cells from endogenous melanin, a commercially available avidin/biotin (ABC)–alkaline phosphatase system utilising a red substrate was used.

3.4.1. Peroxidase–DAB stained tissue sections

Tissue sections, pre-treated with periodic acid and sodium borohydride to block endogenous peroxidase activity (Heydermann and Neville, 1977), were incubated in 5% bovine albumin (Sigma, Poole, UK) in PBS for 15 min. Affinity-purified rabbit IgG anti-SMCP (2.5 μg ml^{-1}) (Huntley et al., 1986) was added for 1 h, prior to washing three times in PBS. Goat anti-rabbit IgG–horseradish peroxidase (2.5 μg ml^{-1}; Sigma), cross-adsorbed against sheep IgG, was then added for 1 h; the section was washed three times in PBS and peroxidase activity was revealed with DAB. As a control, a 1/500 dilution of normal rabbit serum was substituted for antibody.

3.4.2. Vectastain® ABC–alkaline phosphatase (Vector Laboratories, Peterborough, UK) stained tissue sections

Tissue sections mounted on Vectabond® (Vector Laboratories) prepared slides were treated with periodic acid and sodium borohydride as described above. Sections were incubated with normal goat serum for 20 min to reduce background staining. Rabbit anti-SMCP antibody (2.5 μg ml^{-1}) was added for 30 min, and after washing three times in PBS a biotinylated goat anti-rabbit secondary antibody was applied. Alkaline phosphatase activity was visualised with a red substrate (Vector® Red; Vector Laboratories) to which levamisole had been added. Control sections consisted of a 1/500 dilution of normal rabbit serum or PBS in place of primary antibody.

3.5. Cell counts

Tissues were examined using a Leitz Wetzler Dialux 20 EB microscope at ×500 magnification. For non-mucosal tissues, graticule counts were started from a known point of reference so that the same relative area could be counted on both the toluidine blue and anti-SMCP stained sections. Cells were enumerated in a similar manner in mucosal tissues, cell counts within the lamina propria being spread between the basement membrane and epithelium. A minimum of 50 successive fields was counted on each of two adjacent sections stained with toluidine blue or with anti-SMCP. Four pairs of sections were examined for each tissue.

3.6. Enzyme-linked immunosorbent assay (ELISA) for SMCP

3.6.1. Sample preparation

Samples were collected on ice, weighed and stored at −70°C. After thawing, five volumes of ice cold 20 mM Tris/HCl buffer pH 7.5 containing 1.5 M NaCl were added per tissue volume and the tissue homogenised using a Silverson heavy duty laboratory mixer emulsifier. After centrifugation at 800 g, the supernatant fraction was removed and stored at −70°C prior to assay.

3.6.2. ELISA method

The double antibody sandwich ELISA for SMCP is fully described elsewhere (Huntley et al., 1987).

3.7. Statistical analysis

The mean numbers of toluidine blue and SMCP positive cells in each tissue ($n = 4$) were compared using the Student's t-test and for all tissues using linear regression analysis. The number of SMCP positive cells was compared to respective tissue SMCP concentrations (μg SMCP g^{-1} wet tissue) by the use of linear regression analysis.

4. Results

The mean toluidine blue and SMCP mast cell counts, and respective tissue SMCP concentrations, are given in Table 1. The highest mast cell counts (226 mm^{-2} with toluidine blue and 214 mm^{-2} with anti-SMCP), and the highest concentration of SMCP (20 μg SMCP g^{-1} wet tissue) were in the thymus which contained diffusely scattered focal accumulations of cells, with a predominantly perivascular distribution (Fig. 1). The gastrointestinal tissues as a group had relatively high cell counts with both techniques (Fig. 2), and comparatively high tissue SMCP concentrations (Table 1). Despite appreciable numbers of mast cells determined by both histochemical (Fig. 3) and immunohistochemical means, SMCP was not detected in tracheal homogenates by ELISA (Table 1). The cells in this instance were chiefly subepithelial in location.

Table 1
Tissue cell counts and SMCP concentrations

Tissue	*n*	Number of toluidine blue positive cells per mm^2 of tissue (mean ± sem)	Number of SMCP positive cells per mm^2 of tissue (mean ± sem)	Tissue SMCP concentration (μg SMCP) per g of wet tissue (mean ± sem)
Gastrointestinal system				
Abomasum	4	81.9 ± 47.6	90.5 ± 53.6	0.18 ± 0.07
Duodenum	3	80.5 ± 44.9	83.2 ± 34.9	1.3 ± 0.7
Jejunum	4	168.1 ± 26.4	154.9 ± 26.0	1.2 ± 0.4
Ileum	4	126.0 ± 42.2	128.1 ± 43.8	3.3 ± 1.6
Colon	4	65.5 ± 29.1	55.7 ± 23.1	0.6 ± 0.2
Respiratory system				
Trachea	3	19.7 ± 3.5	22.1 ± 4.6	0.0 ± 0.0
Bronchus	3	24.5 ± 3.8	23.8 ± 4.3	0.05 ± 0.05
Bronchrial lymph node	4	61.8 ± 23.7	60.6 ± 20.9	0.12 ± 0.05
Lung	4	50.0 ± 17.3	51.3 ± 18.3	0.11 ± 0.03
Other tissues				
Thymus	3	225.7 ± 23.4	214.4 ± 3.6	19.7 ± 9.3
Spleen	4	73.8 ± 21.8	57.8 ± 13.5	0.07 ± 0.02
Liver	4	80.8 ± 11.8	13.2 ± 1.5	0.005 ± 0.005
Skin	4	42.2 ± 6.0	0.9 ± 0.5	0.02 ± 0.01
Mesenteric lymph node	4	138.2 ± 37.1	134.4 ± 45.8	0.3 ± 0.1

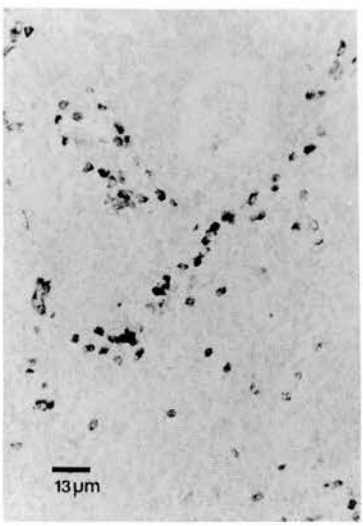


Fig. 1. Toluidine blue stained section of ovine thymus. Note large numbers of darkly stained mast cells focally distributed throughout the section.

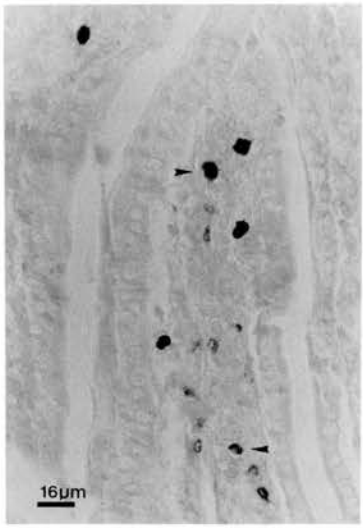


Fig. 2. Toluidine blue stained section of ovine jejunum. Numerous mast cells are present.

There were fewer SMCP positive cells in comparison to toluidine blue positive cells in both skin ($P < 0.01$) and liver ($P < 0.02$). No statistically significant differences were observed for the other individual tissues examined. The anti-SMCP reactive dermal cells were morphologically identical to mast cells (well-granulated mononuclear cells). Dermal basophils were infrequently observed. Their distinct polymorphonuclear appearance and smaller, finer granules allowed them to be differentiated histologically from mast cells. In the liver, far fewer cells (16%) reacted with antibody than with toluidine blue. Morphological examination suggested that these cells were likely to be large granulated lymphocytes.

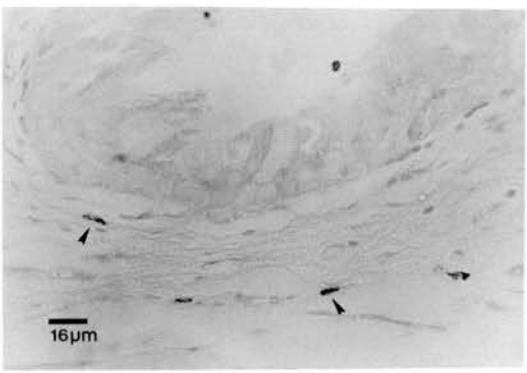


Fig. 3. Toluidine blue stained section of ovine trachea. Note predominantly subepithelial location of mast cells.

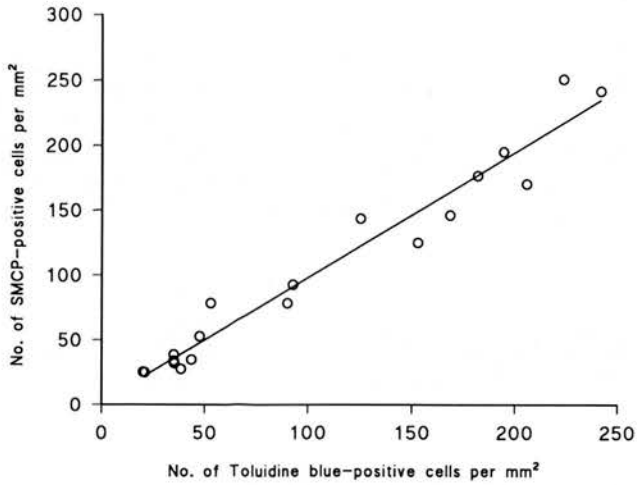


Fig. 4. Comparison of the number of toluidine blue and SMCP positive mast cells per mm^2 in the gastrointestinal tract (abomasum, duodenum, jejunum, ileum, colon). Each point represents the counts for sequential sections from an individual tissue ($r = 0.98$, $r^2 = 0.96$, $P < 0.001$).

There was a positive correlation between toluidine blue and SMCP mast cell counts ($r^2 = 0.96$, $P < 0.001$; Fig. 4) and between SMCP cell counts and tissue SMCP concentrations ($r^2 = 0.30$, $P < 0.02$; Fig. 5) from gastrointestinal tissues.

When the number of cells stained by toluidine blue from all tissues except liver and skin was compared to the number of SMCP stained cells, there was a high degree of

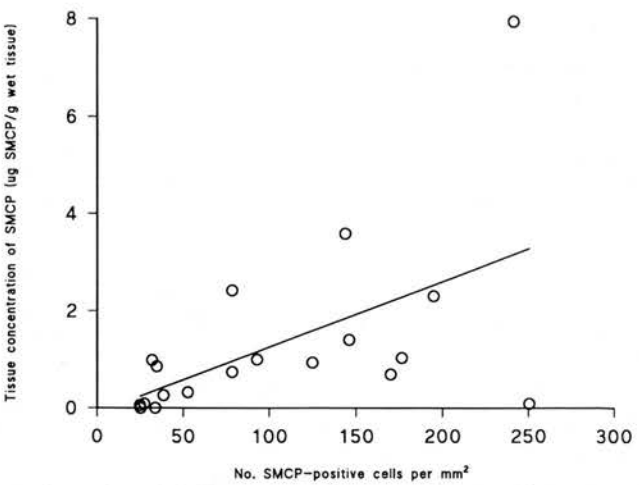


Fig. 5. Comparison of the number of SMCP positive cells per mm^2 versus the concentration ($\mu g\ g^{-1}$ wet tissue) of SMCP in the gastrointestinal tract (abomasum, duodenum, jejunum, ileum, colon). Each point represents an individual tissue sample ($r = 0.55$, $r^2 = 0.30$, $P < 0.02$).

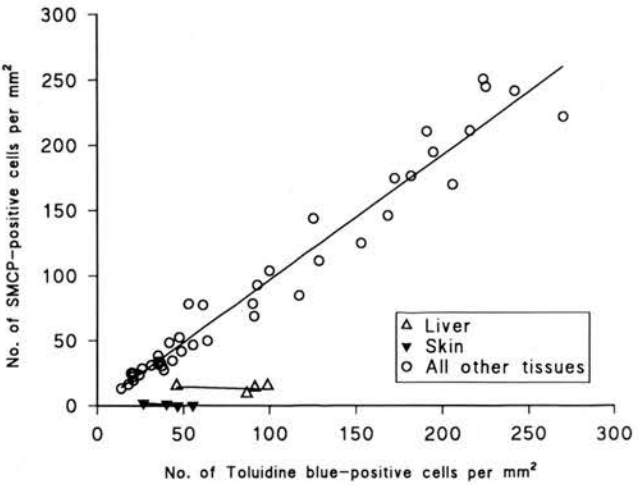


Fig. 6. Comparison of the numbers of toluidine blue and SMCP positive mast cells per mm^2 for all tissues. The regression line is calculated including all tissues except liver and skin ($r = 0.98$, $r^2 = 0.96$, $P < 0.001$). Each point represents an individual tissue sample.

correlation ($r^2 = 0.96$, $P < 0.001$; Fig. 6), in contrast to the finding in skin and liver. A statistically significant correlation was observed between overall SMCP cell counts and concentrations of SMCP in all of the tissues (excluding trachea) ($r^2 = 0.23$, $P < 0.001$).

5. Discussion

Mast cells were detected with the anti-SMCP antibody in virtually all of the tissues examined, but SMCP positive cells were scarce in skin. This indicates the presence of two distinct mast cell populations in sheep, this finding being in accordance with previous studies demonstrating mast cell proteinase heterogeneity in the rat (Gibson and Miller, 1986) and mouse (Newlands et al., 1993) and in man (Schwartz, 1989), where two major mast cell subclasses have been identified.

However, a few SMCP positive cells were observed in skin, indicating proteinase heterogeneity in the dermal mast cell population. This observation may be analogous to the situation in man, where dermal CTMC can be divided into two subpopulations based on the presence or absence of a chymase (Schwartz, 1989). On the basis of cellular morphology, the anti-SMCP antibody did not appear to react with basophils. However, because of the difficulty in distinguishing basophils and mast cells in tissue sections and the apparent paucity of the former in tissues, the possibility that antibody to SMCP cross-reacts with basophils cannot be discounted.

The presence in the liver of a relatively large population of cells putatively identified as large granular lymphocytes was noted. However, careful examination indicated that

these cells did not contribute to the toluidine blue positive cell population in other tissues as stained cells were typically well-granulated and mononuclear, suggesting a mast cell rather than a basophil or lymphocyte lineage. Moreover, these tissues (with the exception of skin) had a high correlation between cell numbers detected with toluidine blue and with antibody to SMCP ($r^2 = 0.96$, $P < 0.001$).

The presence of high numbers of thymic mast cells has been reported previously during studies in rats, although their role remains obscure (Huntley et al., 1993). However, since mast cells produce a wide range of cytokines (Burd et al., 1989; Gordon and Galli, 1990), it is tempting to speculate that mast cell derived cytokines may modulate or augment T-cell responses. Certainly, the role and function of ovine thymic mast cells warrants further study.

Despite there being appreciable numbers of mast cells identified both histochemically and immunohistochemically in trachea, no SMCP could be detected in tracheal homogenates. It is possible that this indicates the presence of a distinct proteinase that is able to cross-react with the anti-SMCP antibody in paraformaldehyde-fixed tissue sections, but is refractory to detection by the ELISA method. Alternatively, it is known that both serum and lymph contain 'factors' that may interfere with SMCP–antibody interactions (Huntley et al., 1987), these possibly being α_2-macroglobulin or serpins (Huntley, 1991). It can be speculated that high inhibitor content in tracheal tissue may have prevented determination of SMCP by ELISA, although immunohistochemical identification in fixed tissue was still possible. This interesting anomaly warrants further investigation.

Previous studies have shown a correlation between the number of gastrointestinal mast cells detected with anti-SMCP and the tissue content of SMCP (Huntley, 1991). On the basis of these results, and the finding that this proteinase was released systemically into blood and lymph following nematode challenge in immune sheep (Huntley et al., 1987), SMCP was considered an 'MMC' enzyme and analogous to RMCP-II in the rat (Gibson et al., 1987). The present investigation extends these findings, with significant correlations ($r^2 = 0.28$, $P < 0.001$) between SMCP tissue content and mast cell numbers in all tissues and organs except skin and liver.

The results of this study indicate that SMCP is present in all mast cells in most of the tissues studied, with the exception of skin. These dermal mast cells presumably contain a proteinase which is antigenically distinct from SMCP and the sheep is, therefore, similar to other species studied in that there is heterogeneity of granule proteases. However, there are also species differences which may have important implications in terms of function and activity of these cells. For instance, in the rat RMCP-I-containing CTMC predominate in a variety of connective tissues including skin and are widely distributed throughout these organs. Although RMCP-II-containing MMC are found principally in the gastrointestinal tract, they are not confined to this tissue, and indeed can be located in non-mucosal sites (Huntley et al., 1993). The distribution of ovine mast cell subsets is apparently different to the rat, with clearly defined anatomical locations for the mast cell proteinase populations. Thus, there exists diversity between species in mast cell proteinase heterogeneity and expression, and further studies involving the isolation of ovine dermal mast cell proteinase(s) are required for a full evaluation of mast cell heterogeneity in the sheep.

Acknowledgements

G. Sture is the grateful recipient of a Wellcome Trust Veterinary Training Scholarship (035589/1 · 5B). J. Huntley and A. MacKellar were supported by funding from the Scottish Office Agriculture and Fisheries Department.

References

Burd, P.F., Rogers, H.W., Gordon, J.R., Martin, C.A., Jayaraman, S., Wilson, S.D., Dvorak, A.M., Galli, S.J. and Dorf, M.E., 1989. IL-3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. J. Exp. Med., 170: 245–258.

Enerback, L., 1966. Mast cells in rat gastrointestinal mucosa. I. Effect of fixation. Acta Pathol. Microbiol. Scand., 66: 289–302.

Galli, S.J., 1990. New insights into 'The riddle of the mast cells': microenvironmental regulation of mast cell development and phenotypic heterogeneity. Lab. Invest., 62: 5–33.

Gibson, S. and Miller, H.R., 1986. Mast cell subsets in the rat distinguished immunohistochemically by their content of serine proteinases. Immunology, 58: 101–104.

Gibson, S., MacKellar, A., Newlands, G. and Miller, H.R.P., 1987. Phenotypic expression of mast cell granule proteinases. Distribution of mast cell proteinases I and II in the rat digestive system. Immunology, 62: 621–627.

Gordon, J.R. and Galli, S.J., 1990. Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. Nature, 346: 274–276.

Heydermann, E. and Neville, A.M., 1977. A shorter immunoperoxidase technique for the demonstration of carcino-embryonic antigen and other cell products. J. Clin. Pathol., 30: 138–140.

Huntley, J.F., 1991. Mast cells and intestinal nematodiasis. Ph.D. Thesis, The University of Edinburgh.

Huntley, J.F., Gibson, S., Knox, D. and Miller, H.R.P., 1986. The isolation and purification of a proteinase with chymotrypsin-like properties from ovine mucosal mast cells. Int. J. Biochem., 18: 673–682.

Huntley, J.F., Gibson, S., Brown, D., Smith, W.D., Jackson, F. and Miller, H.R.P., 1987. Systemic release of a mast cell proteinase following nematode infections in sheep. Parasite Immunol., 9: 603–614.

Huntley, J.F., MacKellar, A., Newlands, G.F., Irvine, J. and Miller, H.R.P., 1990. Mapping of the rat mast cell granule proteinases RMCPI and II by enzyme-linked immunosorbent assay and paired immunofluorescence. APMIS, 98: 933–944.

Huntley, J.F., MacKellar, A. and Miller, H.R.P., 1993. Altered expression of mast cell proteases in the rat. Quantitative and immunohistochemical analysis of the distribution of rat mast cell proteases I and II during helminth infection. APMIS, 101: 953–962.

Knox, D.P. and Huntley, J.F., 1987. Classification of sheep abomasal mucosal mast cell proteinase as a serine endopeptidase (EC 3.4.21). Int. J. Biochem., 20: 193–195.

Lagunoff, D. and Pritzl, P., 1976. Characterization of rat mast cell granule proteins. Arch. Biochem. Biophys., 173: 554–563.

Miller, H.R.P., Jackson, F., Newlands, G. and Appleyard, W.T., 1983. Immune exclusion, a mechanism of protection against the ovine nematode *Haemonchus contortus*. Res. Vet. Sci., 35: 357–363.

Miller, H.R.P., Huntley, J.F., Newlands, G.F.J., MacKellar, A., Irvine, J., Haig, D.M., MacDonald, A., Lammas, A.D., Wakelin, D. and Woodbury, R.G., 1989. Mast cell granule proteases in mouse and rat: a guide to mast cell heterogeneity and activation in the gastrointestinal tract. In: S. Galli and K.F. Austen (Editors), Mast Cell and Basophil Differentiation and Function in Health and Disease. Raven Press, New York, pp. 81–91.

Newlands, G.F.J., Huntley, J.F. and Miller, H.R.P., 1984. Concomitant detection of mucosal mast cells and eosinophils in the intestines of normal and *Nippostrongylus*-immune rats. Histochemistry, 81: 585–589.

Newlands, G.F., Gibson, S., Knox, D.P., Grencis, R., Wakelin, D. and Miller, H.R., 1987. Characterization and mast cell origin of a chymotrypsin-like proteinase isolated from intestines of mice infected with *Trichinella spiralis*. Immunology, 62: 629–634.

Newlands, G.F.J., Gibson, S., Knox, D.P., Grencis, R., Wakelin, D. and Miller, H.R.P., 1993. Biochemical and immunological characterisation of multiple glycoforms of mouse mast cell protease 1: comparison with an isolated murine serosal mast cell protease (MMCP-4). Biochem. J., 294: 127–135.

Reynolds, D.S., Stevens, R.L., Lane, W.S., Carr, M.H., Austen, K.F. and Serafin, W.E., 1990. Different mouse mast cell populations express various combinations of at least six distinct mast cell serine proteases. Proc. Natl. Acad. Sci. USA, 87: 3230–3234.

Schechter, N.M., Choi, J.K., Slavin, D.A., Deresienski, D.T., Sayama, S., Dong, G., Lavker, R.M., Proud, D. and Lazarus, G.E., 1986. Identification of a chymotrypsin-like proteinase in human mast cells. J. Immunol., 137: 962–970.

Schwartz, L.B., 1989. Heterogeneity of mast cells in humans. In: S.J. Galli and K.F. Austen (Editors), Mast Cell and Basophil Differentiation and Function in Health and Disease. Raven Press, New York, pp. 93–106.

Woodbury, R.G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D. and Neurath, H., 1978. An intracellular serine protease from rat skeletal muscle in the chymotrypsin-like enzymes of mast cells. Proc. Natl. Acad. Sci. USA, 75: 5311–5313.

Veterinary immunology and immunopathology

Submission of manuscripts: Manuscripts should be submitted in triplicate to The Editorial Office of *Veterinary Immunology and Immunopathology*, P.O. Box 181, 1000 AD Amsterdam, Netherlands. Contributors from the Americas can submit papers direct to the North American Editor-in-Chief, Professor S. Krakowka, Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210, USA.

Electronic manuscripts: Electronic manuscripts have the advantage that there is no need for the rekeying of text, thereby avoiding the possibility of introducing errors and resulting in reliable and fast delivery of proofs.
For the initial submission of manuscripts for consideration, hardcopies are sufficient. For the processing of **accepted papers**, electronic versions are preferred. After **final acceptance**, your disk plus two final and exactly matching printed versions should be submitted together. Double density (DD) or high density (HD) diskettes (3.5 or 5.25 in) are acceptable. It is important that the file saved is in the native format of the word processor program used. Label the disk with the name of the computer and word processing package used, your name, and the name of the file on the disk. Further information may be obtained from the Publisher.

Authors in Japan please note: Upon request, Elsevier Science Japan will provide authors with a list of people who can check and improve the English of their paper (*before submission*). Please contact our Tokyo office: Elsevier Science Japan, 20–12 Yushima 3-chome, Bunkyo-ku, Tokyo 113. Tel: (03) 3833 3821; fax: (03) 3836 3064.

All questions arising after acceptance of the manuscript, especially those relating to proofs, should be directed to Elsevier Editorial Services, Mayfield House, 256 Banbury Road, Oxford OX2 7DH, UK. Tel: +44 (0) 1865 314900; fax: +44 (0) 1865 314990.

Advertising information: Advertising orders and enquiries may be sent to: Elsevier Science B.V., Advertising Department, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. Tel: (+3120) 4853795. Fax (+3120 4853810. Courier shipments to street address: Molenwerf 1, 1014 AG Amsterdam, The Netherlands. In the UK: TG Scott & Son Ltd., attn. Vanessa Bird, Portland House, 21 Narborough Rd., Cosby, LE9 5TA, UK. Tel. (0116)2750-521/2753-333; Fax: (0116)2750-522. In the USA and Canada: Weston Media Associates, attn. Daniel Lipner, P.O. Box 1110, Greens Farms, CT 06436-1110. USA. tel. (203)261-2500; fax (203)261-0101.

***Veterinary Immunology and Immunopathology* has no page charges**

For a full and complete Guide for Authors please refer to *Veterinary Immunology and Immunopathology*, Vol. 43, No. 4, pp. 414–420

 0165-2427/95/$09.50

♾ The paper used in this publication meets the requirements of ANSI/NISO 239.48-1992 (Permanence of Paper).

Printed in The Netherlands

US mailing notice: *Veterinary Immunology and Immunopathology* (ISSN 0165-2427) is published monthly by Elsevier Science B.V. (Molenwerf 1, Postbus 211, 1000 AE Amsterdam). Annual subscription price in the USA is US$1093.00 (valid in North, Central and South America only), including air speed delivery. Second class postage rate is paid at Jamaica, NY 11431.
USA POSTMASTERS: send address changes to *Veterinary Immunology and Immunopathology*, Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003.
AIRFREIGHT AND MAILING in the USA by Publication Expediting Inc., 200 Meacham Avenue, Elmont, NY 11003.